Requested Patent: WO0118237A1

Title: INACTIVE ENZYMES AS NON-CONSUMING SENSORS ;

Abstracted Patent: WO0118237 ;

Publication Date: 2001-03-15 ;

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Application Number: WO2000US24846 20000911 ;

Priority Number(s): US19990153068P 19990910; US20000203605P 20000512 ;

IPC Classification: C12Q1/26; C12Q1/32; G01N33/573 ;

Equivalents: AU7367300 ;

ABSTRACT:

Inactive enzymes such as apo-enzymes are used as non-consuming sensors, which bind with analytes without destroying the analytes. These sensors can be used in methods, assays or kits to measure analyte concentration. Inactive forms of various enzymes such as glucose oxidase, glucose dehydrogenase, or lactate dehydrogenase which binds to glucose or lactate can be used as biosensors of glucose or lactate.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18237 A1

(51) International Patent Classification[7]: C12Q 1/26, 1/32, G01N 33/573

(21) International Application Number: PCT/US00/24846

(22) International Filing Date:
11 September 2000 (11.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/153,068 10 September 1999 (10.09.1999) US
60/203,605 12 May 2000 (12.05.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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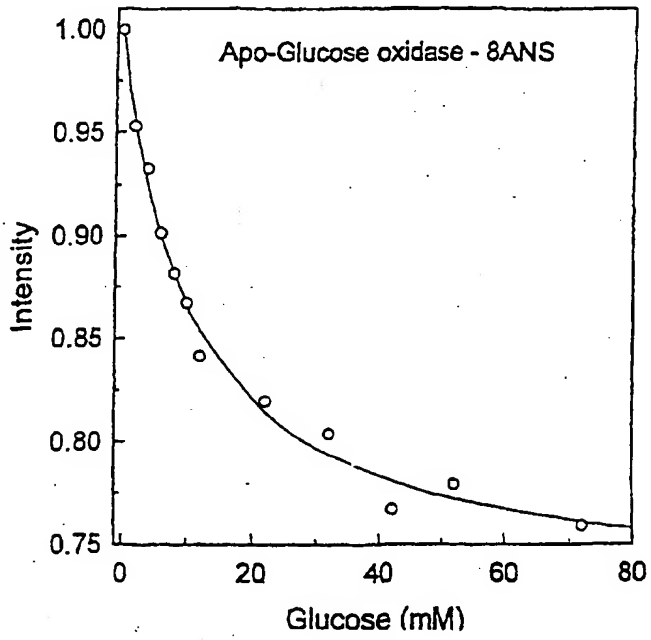
(54) Title: INACTIVE ENZYMES AS NON-CONSUMING SENSORS



Glucose-dependent emission intensity of 1,8-ANS bound to apo-glucose oxidase. Excitation at 325 nm, emission at 480 nm.

(57) Abstract: Inactive enzymes such as apo-enzymes are used as non-consuming sensors, which bind with analytes without destroying the analytes. These sensors can be used in methods, assays or kits to measure analyte concentration. Inactive forms of various enzymes such as glucose oxidase, glucose dehydrogenase, or lactate dehydrogenase which binds to glucose or lactate can be used as biosensors of glucose or lactate.

WO 01/18237 A1

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ASSAYING ANALYTES USING INACTIVE ENZYMES

Cross Reference to Related Applications

This application claims priority on U.S. Provisional Applications 60/153,068, filed September 10, 1999, and 60/203,605, filed May 12, 2000, which are incorporated herein by reference in their entirety.

Government Interests

The invention disclosed herein was made with Government support under NIH Grant No. RR-08119. Accordingly, the U.S. Government has certain rights in this invention.

Background of the Invention

(1) Field of the Invention

The present invention relates to an assay for the measurement of analytes such as glucose or lactate using an inactive enzyme that binds to the analyte such as an apo-enzyme. Such apo-enzymes include inactive forms of glucose oxidase, glucose dehydrogenase, lactate oxidase or lactate dehydrogenase. Further, the invention more specifically relates to the measurement of such analytes, such as by fluorescent measurement.

(2) Description of the Related Art

The measurement of biochemical analytes is important for health care. Blood glucose is a clinically important analytes for diabetic health care, because close control of blood glucose is necessary to avoid the long term health effects of diabetes. Close regulation of blood glucose is essential for diabetics to minimize the long term adverse health consequences of diabetes. The Diabetes Control and Complications Trial Research Group (1997) *Diabetes* 4, 271- 286; The Diabetes Control and Complications Trial Research Group (1993), *N. Engl. J Med.* 329, 977-986. Measurement of blood glucose requires a finger stick, which is done by most diabetics. As a result there is a substantial worldwide effort to develop non-invasive and minimally invasive methods for frequent or continuous monitoring of blood glucose. A wide variety of methods have been tested, including optical rotation (Rabinovitch, B., *et al.* (1982) *Diabetes Care* 5(3), 254-258; March, W. F., *et al.* (1982) *Trans. Am. Soc. Artif Intern. Organs* 28, 232-235), near infrared

absorbance and Raman scattering (Amato, I. (1992) *Science* 258, 892-892. Robinson, M. R., *et al.* (1992) *Clin. Chem.* 38(9), 1618-1622; Yu, N-T., *et al.* (1996) *J Biomed. Optics* 1(3), 280-288) and the design and synthesis of glucose-specific fluorescence probes (James, T. D., *et al.* (1996) *Chem. Commun.* pp. 705-706; James, T. D., *et al.* (1997) *Chem. Commun.* pp. 71-72). Because of its simplicity and sensitivity, fluorescence has been extensively evaluated for use in glucose sensing. Most of these studies involving fluorescence used the binding of a glucose-specific protein such as Concanavalin A dextran to glucose or some polysaccharide by resonance energy transfer. This work has its origins with the studies of Schultz and co-workers (Schultz, J. S., *et al.* (1979) *Biotech. Bioeng. Symp.* 9,65-71; Schultz, J., *et al.* (1982) *Diabetes Care* 5(3), 245-253; Meadows, D., *et al.* (1988) *Talanta* 35(2), 145-150) who developed a competitive glucose assay which does not require substrates and does not consume glucose. This assay used fluorescence resonance energy transfer (RET) between a fluorescence donor and an acceptor, each covalently linked to concanavalin A (ConA) or dextran. In the absence of glucose the binding between ConA and dextran resulted in a high RET efficiency. The addition of glucose resulted in its competitive binding to ConA, ConA displacement of ConA from the labeled dextran, and a decrease in the RET efficiency.

In this laboratory, we also studied the ConA-dextran system for glucose sensing. In our case we chose to use fluorescence decay times in place of the fluorescence intensities. We chose lifetime-based sensing because the lifetimes are mostly independent of the total intensity. Szmacinski, H., *et al.* (1994) "Lifetime-based sensing," in *Topics in Fluorescence Spectroscopy* (J. R. Lakowicz, ed.), Plenum Press, New York, Vol. 4., pp. 295-334; Szmacinski, H., *et al.* (1995) *Sensors and Actuators* B 29, 15-24; Lippitsch, M. E., *et al.* (1997) *Sensors and Actuators* B 38-39, 96-102.

We used ConA-dextran to develop lifetime sensors using both visible and NIR fluorophores (Lakowicz, J. R., *et al.* (1993) *Anal. Chim. Acta* 271, 155-164; Tolosa, L., *et al.* (1997) *Sensors and Actuators*, 45, 93-99) and a long-lifetime metal-ligand complexes as the donor (Tolosa, L., *et al.* (1997) *Anal. Biochem.* 250, 102-108). While the ConA-dextran system worked in the laboratory, the sensors were only partially reversible, and they become increasingly irreversible with time following mixing of the ConA and dextran. We often observed precipitation. We reasoned these effects were due to binding of the multivalent ConA with the multivalent dextran and subsequent cross-linking. To circumvent this problem we developed a sensor based on ConA and a small protein labeled

with a single covalently attached glucose residue (Tolosa, L., *et al. supra*), but this sensor also proved to be only partially reversible.

To circumvent the difficulties of lack of reversibility we decided to explore the use of proteins which bind glucose, such as those present in bacteria. We reasoned that a monovalent glucose binding protein interacting with a monomeric sugar would be completely reversible.

In our initial studies we used the glucose-galactose binding protein (GGBP) from *E. coli*, which was labeled with a fluorophore at a single genetically cysteine residue at position 26. Tolosa, L., *et al.* (1999) *Anal. Biochem.* 267, 114-120. This labeled protein displayed spectral changes in response to micromolar concentrations of glucose.

We also extended the use of glucose sensing with other protein which binds glucose. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (GO) is an oxidoreductase enzyme that catalyzes the conversion of β-D-glucose and oxygen to D-glucono-1,5-lactone and hydrogen peroxide, and as probes to monitor the concentrations of glucose. Lakowicz, J. R., *et al.* (1999) *Anal. Biochem.* 267:397-405. This GO is a flavoprotein, highly specific for β-D-glucose (Manstein, D. J., *et al.* (1986) *Biochemistry* 25,22, 6807-6816) and is widely used to estimate glucose concentration in blood or urine samples through the formation of colored dyes by the hydrogen peroxide produced in the reaction. Teng, F. Y., *et al.* (1995) *J Soc. Gynecol Invest.* 2, 618-622; Miyasaka, T., *et al.* (1997) *ASAIO J.* 43, M505-M509. Because glucose is consumed, this enzyme cannot be used as a reversible sensor.

In addition, we examined the use of a thermostable glucose dehydrogenase as a glucose sensor. In general, biotechnological applications of enzymes are often hampered by their low stability to heat, pH, organic solvents, and proteolysis. Sthal, S. (1993) In *Thermostability of Enzymes* (Gupta M. N. ed pp 45-74), Springer-Verlag, Berlin; Shoichet, B.K., *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92, 452-456. Many attempts have been made to improve the stability of current commercial enzymes, as well as to establish guidelines for improving the stability of protein and enzymes. Martinek, K., *et al.* (1993) In *Thermostability of enzymes* (Gupta M.N. ed pp 45-74), Springer-Verlag, Berlin; Mozhaev, VV, *et al.* (1990) *Adv Drug Deliv Rev* 4, 387-393; Mozhaev, VV, *et al.* (1988) CRC *Crit. Rev. Biochem* 25, 235-281. Enzymes isolated from thermophilic sources are natural examples of stable biomolecules. In fact, thermophilic enzymes are not only stable

and active at high temperature, but they are often stable in the presence of organic solvents and detergents. D'Auria, *et al. (1997) Biochem J* 323, 833-840.

Glucose dehydrogenase (GD) from the thermoacidophilic archaeon *Thermoplasma acidophilum* is a tetramer of about 160 kDa composed of four similar subunits of about 40 kDa each. The enzyme shows a Km value of 10 mM for glucose, and it is resistant to high temperatures and organic solvents. At 55°C, full activity is retained after 9 hours, and at 75°C the half-life is approximately 3 hours. Moreover, incubation of the enzyme for up 6 hours at room temperature with 50% (v/v) methanol, acetone or ethanol without any appreciable loss of activity. Smith, L.D., *et al.* (1989) *Biochem J 261,* 973-977.

Aside from glucose, there is considerable medical interest in measurements of blood lactate. A resting adult produces approximately 1.3 moles of L-lactate per day. L-lactate, referred to here as lactate, is removed by gluconogenesis in the liver and to a smaller extent by oxidation in skeletal muscle and the renal cortex. Burtis, C. A., *et al.* (1999) *Tietz Textbook of Clinical Chemistry,* W.B. Saunders Co., London, pp. 1917. Normal resting concentrations of blood lactate range from 0.36 to 0.75 mM at rest with somewhat higher values of 0.36 to 1.7 mM for hospitalized patients. *Id.* Elevated concentrations of blood lactate are indicators of a considerable number of medical conditions. As examples, serum lactate levels are predictive of survival in children after open heart surgery (Siegel, L. B., *et al.* (1996) *Intensive Care Med.* 22:1418-1423.), mortality in ventilated infants (Deshpande, S. A., *et al.* (1997) *Disease in Childhood* 76: F15-F20) and may be preferable to pH for evaluating fetal intraparteum asphyxia (Westgren, M., *et al. British Journal of Obstetrics and Gynaecology* 105:29-33). In adults elevated blood lactate can predict multiple organ failure and death in patients with septic shock (Bakker, J., et al. (1996) *American Journal of Surgery* 171:221-226) and the function of newly transplanted livers (DeGasperi, A., *et al.* (1997) *Int. J. Clin, Lab. Res.* 27:123-128). Lactic acidosis is also known to accompany decreased tissue oxygenation, hypovolemic, left ventricular failure and drug toxicity (Burtis, C. A., *supra*). Measurement of blood lactate is also valuable for monitoring the results of exercise and athletic performance (Palleschi, G., *et al.* (1990) *Med & Biol. Eng & Comput.* 28-B25-B28) D-lactate is not produced by humans, and is not found in the blood except in the presence of unusual intestinal bacteria.

While blood lactate is a useful diagnostic indicator, its use is hindered by the time required for a lactate determination. Even under favorable conditions a lactate measurement typically takes 30 minutes or longer, which is too long for many clinical

decisions, particularly with critically ill patients (Detry, B., *et al.* (1998). *Eur Respir J.* 11:183-187). Lactate determinations are typically performed by enzymatic oxidation to pyruvate by lactate dehydrogenase (LDH) or lactate oxidase, followed by detection of NADH or H_2O_2, respectively (Burtis, C. A., *supra*; Palmisano, F., *et al.*; (1996) *Biosensors & Bioelectronics* 11:419-425; DeMarcos, S., *et al.* (1997) *The Analyst* 122:355-359; Marzouk, S. A., *et al.* (1997) *Anal. Chem.* 69:2646-2652).

Lactate dehydrogenase is a tetramer with a molecular weight of 136,700 ± 2,100 daltons. A number of isozymes are known to occur as mixed tetramers of the muscle and heart isozymes.

Summary of the Invention

The present invention includes a method for assaying a sample suspected of containing an analyte comprising: (a) contacting the sample with a composition comprising an inactive enzyme to form a complex; wherein the analyte is not consumed in the complex, and wherein the enzyme is coupled to the analyte during the contacting step; and (b) measuring the amount or presence of analyte coupled to the enzyme.

Another embodiment of the invention is an assay for assaying a sample suspected of containing an analyte. This assay comprises the sample and an inactive enzyme forming a complex, wherein the analyte reversibly couples to the enzyme, and wherein the inactive enzyme does not consume the analyte.

In a preferred embodiment of the method or assay, the inactive enzyme comprises a label. The label can be an intrinsic or extrinsic label, and preferably an intrinsic label. More preferably, the label is a fluorescent label, a luminescent label, an enzyme label, a radioactive label, or a chemical label, and more preferably a fluorescent label, most preferably 8-anilino-1-naphthalene sulfonic acid (ANS). In the method, the amount or presence of an analyte preferably is measured by emission maxima, emission intensity, spectral shift, energy transfer, anisotropy, polarization, lifetime or wavelength ratio.

The preferred analytes for the method or assay include sugar, preferably glucose, or lactate or a substrate for an oxidase or a dehydrogenase. Preferably these analytes are found in biological samples such as whole blood, serum or plasma. In one embodiment, the inactive enzyme is a mutated enzyme, an apo-enzyme or an inhibited enzyme and is preferably an apo-enzyme. The preferred inactive enzyme is an apo-enzyme such as apo-glucose oxidase, apo-glucose dehydrogenase, apo-lactate dehydrogenase or apo-lactate

oxidase. Preferably the enzyme is produced from a thermophilic organism. The assay can be a homogenous assay or a heterogeneous assay, and preferably the assay is a homogenous assay.

The invention also include a kit for assaying a sample suspected of containing an analyte. The kit includes a composition having an inactive enzyme wherein the analyte is not consumed upon measurement. The kit can be used as an assay to measure analytes such as sugar, and more preferably glucose, or lactate. In one embodiment, the inactive enzyme is preferably an apo-enzyme in which a cofactor has been removed therefrom, such as apo-glucose oxidase, apo-glucose dehydrogenase, apo-lactate dehydrogenase or apo-lactate oxidase. In another embodiment, the enzyme is produced from a thermophilic organism. In a preferred embodiment, the inactive enzyme comprises a label. The label can be an intrinsic or extrinsic label, and preferably an intrinsic label. More preferably, the label is a fluorescent label, a luminescent label, an enzyme label, a radioactive label, or a chemical label, and more preferably a fluorescent label, most preferably 8-anilino-1-naphthalene sulfonic acid (ANS). The sample is preferably a biological sample. In a preferred embodiment, the kit also includes instructions for measuring the amount or presence of the analyte in a sample.

Brief Description of the Drawings

Figure 1. Absorption and emission spectra of apo-glucose oxidase. Excitation at 298 nm. The protein concentration was 0.05 mg/ml.

Figure 2. Emission spectra 5 μM 1,8-ANS in the presence of 3 μM apo-glucose oxidase. Excitation at 325 nm.

Figure 3. Glucose-dependent emission intensity of 1,8-ANS bound to apo-glucose oxidase. Excitation at 325 nm, emission at 480 nm.

Figure 4. Frequency-domain intensity decays of 1,8-ANS-apo-glucooxidase in the presence of increasing concentrations of glucose. Excitation was 335 nm (DCM dye laser), emission was observed through interference filter 535/50 nm and two Coming 3-71 cutoff filters.

Figure 5. Glucose-dependent lifetimes and pre-exponential factors from the lifetime-global analysis.

Figure 6. Effect of glucose on the mean decay time of 1,8-ANS-labeled apo-glucose oxidase.

Figure 7. Polarization sensing. Simulations of the expected changes in polarization for different values of k.

Figure 8. ANS-labeled glucose dehydrogenase (GD) fluorescence intensity in the presence of different concentrations of acetone. [GD] = 3 μM. [ANS] = 4 μM. The excitation was at 370 nm, and the emission was monitored at 510 nm.

Figure 9. Emission spectra of ANS-labeled GD in the presence of 3% acetone and at different concentrations of glucose. [GD] = 3 μM. [ANS] = 4 μM. Increase of glucose concentration over 70 mM does not introduce further changes in fluorescence intensity.

Figure 10. Emission spectra of ANS-labeled GD in the presence of 15% acetone (top) and 30% acetone (bottom) and at different concentrations of glucose. [GD] = 3 μM. [ANS] = 4 μM. Increase of glucose concentration over 70 mM does not introduce further changes in fluorescence intensity.

Figure 11. Frequency-domain intensity decay of ANS-labeled GD with 3% acetone in the absence and presence of glucose.

Figure 12. Polarization spectra of ANS-labeled GD in the presence of 3% acetone, and at different concentrations of glucose. Excitation was at 370 nm. [GD] = 3 μM. [ANS] = 4 μM.

Figure 13. Effect of glucose on the polarization of GD in the presence of 3% acetone. The excitation was at 370 nm, and the emission was recorded at 470 nm. [GD] = 3 μM. [ANS] = 4 μM.

Figure 14. Intrinsic fluorescence of LDH in the absence and presence of lactate.

Figure 15. Frequency-domain intensity decay of the intrinsic fluorescence of LDH in the absence and presence of lactate.

Figure 16. Emission spectra of ANS-labeled LDH in the presence of increasing concentrations of lactate.

Figure 17. Relative emission intensity of ANS-labeled LDH in the presence of increasing concentrations of lactate.

Figure 18. Frequency-domain intensity decay of ANS-labeled LDH in the absence and presence of lactate.

Figure 19. Schematic of polarization sensing (top) and simulations of the expected changes in compensation angle for different values of n.

Figure 20. Polarization sensing of lactate bound on the emission intensity of ANS-labeled LDH.

Detailed Description of the Invention

In order to overcome the disadvantages of conventional methods of measuring an enzyme's substrate, the present invention provides a method for measuring the amount or presence of an analyte in a sample suspected of containing the analyte comprising: contacting the sample with a composition comprising an enzyme to form a complex; wherein the analyte is not consumed in the complex, and wherein the enzyme is coupled to the analyte during the contacting step; and measuring the amount or presence of analyte coupled to the inactive enzyme.

In another embodiment, the invention includes a kit for measuring the amount or presence of an analyte in a biological sample. The kit comprises a composition having an inactive enzyme wherein the analyte is not consumed upon measurement.

A further embodiment includes an assay for determining the amount of an analyte in a sample suspected of containing an analyte comprising a sample in combination with an inactive enzyme preferably coupled with a labeling reagent, and wherein the amount or presence of an analyte is measured as a result of the analyte binding with the inactive enzyme.

A sample comprises any fluid containing the analyte which is often a biological sample. Preferably a biological sample is used such as a sample of blood, blood serum, plasma, urine, interstitial fluid, body secretion, tears, saliva, lymphatic or other extract taken from a mammal, preferably a human, to be tested for the presence of an analyte. More preferably the sample is whole blood, plasma, or serum.

An inactive enzyme is an enzyme that retains the ability to bind to a substrate, but that does not consume the substrate, wherein the substrate is the analyte to be measured. In a preferred embodiment, inactive enzymes of the invention include apo-enzymes. In addition, inactive enzymes can include enzymes that are engineered using site-directed mutagenesis. Mutants or analogs of the gene that produces an inactive enzyme may be prepared by the deletion, insertion or substitution of one or more nucleotides of the coding sequence. Techniques for modifying nucleotide sequences, such as site-directed

mutagenesis, are described in, *e.g.*, Sambrook *et al.*, *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*. Further, inactive enzymes can include enzymes that are linked to an uncompetitive inhibitor, which do not prevent analyte binding, but render the enzyme inactive. Preferably, such uncompetitive inhibition results from a covalent reaction with inhibitors. In a preferred embodiment, the enzymes such as dehydrogenases and oxidases are produced from thermophilic organisms. For example, lactate dehydrogenase and lactate oxidase can be produced from thermophilic organisms and analyte binding proteins such as lactate binding proteins can also be produced from normal thermophilic organisms which are stabilized by mutating their amino acid sequence or by immobilization.

An apo-enzyme is an enzyme that does not have a cofactor attached thereto. Apo-enzymes included within the scope of the invention include those enzymes normally attached to cofactors. Such cofactors include, but are not limited to 6-hydroxyDOPA, ammonia, ascorbate, ATP, bile salts, biotin, cadmium, calcium, cobalamin, cobamide coenzymes, cobalt, coenzyme-A, copper, dipyrromethane, dithiothreitol, divalent cation, F420, flavin adenine dinucleotide (FAD), flavin, flavoprotein, flavin mononucleotide (FMN), glutathione, heme, heme-thiolate, iron, iron-molybdenum, iron-sulfur, lipoyl group, magnesium, manganese, molybdenum, monovalent cation, nicotinamide adenine dinucleotide (NAD), NAD(P)H, nickel, potassium, PQQ, protoheme IX, pterin, pyridoxal phosphate, pyruvate, reduced flavin, selenium, siroheme, sodium, tetrahydrofolate, tetrahydropteridine, thiamine pyrophosphate, tryptophan tryptophylquinone (TTQ), tungsten, vanadium, or zinc. Please see Swiss Institute of Bioinformatics, ExPASy Molecular Biology Server, http://www.espasy.ch/cgi-bin/enzyme-search-cf, which is in incorporated herein by reference.

Apo-enzymes within the scope of the invention include but are not limited to those disclosed in Swiss Institute of Bioinformatics, ExPASy Molecular Biology Server, http://www.espasy.ch/cgi-bin/enzyme-search-cf, which is in incorporated herein by reference. Preferably, the apo-enzymes of the invention are inactive and therefore they are coupled to their respective substrates without consuming the substrates and have a spectral change with respect to a substrate-bound apo-enzyme. For example, the apo-enzymes include:

oxidases including amine oxidase (copper-containing), malate oxidase, glucose oxidase, cholesterol oxidase, L-gluconolactone oxidase, pyridoxine 4-oxidase, alcohol

oxidase, choline oxidase, 4-hydroxymandelate oxidase, glycerol-3-phosphate oxidase, xanthine oxidase, thiamine oxidase, L-galactonolactone oxidase, methanol oxidase, D-arabinono-1,4-lactone oxidase, vanillyl-alcohol oxidase, pyruvate oxidase, pyruvate oxidase (CoA-acetylating), dihydroorotate oxidase, protoporphyrinogen oxidase, acyl-CoA oxidase, D-aspartate oxidase, L-amino acid oxidase, D-amino acid oxidase, amine oxidase (flavin-containing), putrescine oxidase, L-glutamate oxidase, cyclohexylamine oxidase, L-lysine oxidase, D-glutamate(D-aspartate) oxidase, L-aspartate oxidase, sarcosine oxidase, (S)-6-hydroxynicotine oxidase, (R)-6-hydroxynicotine oxidase, reticuline oxidase, dimethylglycine oxidase, polyamine oxidase, glutathione oxidase, cellobiose oxidase, oxalate oxidase, carbon-monoxide oxidase, N-methyl-L-amino-acid oxidase, O-aminophenol oxidase;

oxygenases including heme oxygenase (decyclizing), carbon monoxide oxygenase (cytochrome b-561);

dioxygenases including gamma-butyrobetaine, 2-oxoglutarate dioxygenase, procollagen-proline, 2-oxoglutarate-4-dioxygenase, pyrimidine-deoxynucleoside 2'-dioxygenase, procollagen-lysine 5-dioxygenase, thymine dioxygenase, procollagen-proline 3-dioxygenase, trimethyllysine dioxygenase, naringenin 3-dioxygenase, pyrimidine-deoxynucleoside 1'-dioxygenase, hyoscyamine (6S)-dioxygenase, gibberellin 3-beta-dioxygenase, beta-carotene 15,15'-dioxygenase, benzene 1,2-dioxygenase, 3-hydroxy-2-methylpyridinecarboxylate dioxygenase, indole 2,3-dioxygenase, 5-pyridoxate dioxygenase, Cysteine dioxygenase;

mutases including 2-acetolactate mutase, beta-lysine 5,6-aminomutase, D-lysine 5,6-aminomutase, D-ornithine 4,5-aminomutase, leucine 2,3-aminomutase, methylaspartate mutase, methylmalonyl-CoA mutase, 2-methyleneglutarate mutase;

peptidases including beta-Ala-His dipeptidase, Prolyl aminopeptidase, Xaa-Pro aminopeptidase, clostridial aminopeptidase, tryptophanyl aminopeptidase, Cys-Gly dipeptidase, Xaa-Pro dipeptidase, Met-Xaa dipeptidase;

transferases including 5-methyltetrahydrofolate-homocysteine S-methyltransferase, glycerophospholipid acyltransferase (CoA-dependent), [Methionine synthase]-cobalamin methyltransferase (cob(II)alamin reducing), delta(24)-sterol C-methyltransferase, dihydrolipoamide S-acetyltransferase, dihydrolipoamide S-succinyltransferase, methionine S-methyltransferase, polypeptide N-acetylgalactosaminyltransferase, xylosylprotein 4-beta-galactosyltransferase, galactosylxylosylprotein 3-beta-galactosyltransferase,

galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase, leucyltransferase, beta-galactosyl-N-acetylglucosaminylgalactosyl-glucosylceramide-1,3-acetylglucosaminyltransferase, galactosyl-N-acetylglucosaminylgalactosyl-glucosylceramide beta-1,6-N-acetylglucosaminyltransferase, N-acetylneuraminylgalactosylglucosylceramide beta-1,4-N-acetylgalactosaminyltransferase, glucuronylgalactosylproteoglycan beta-1,4-N-acetylgalactosaminyltransferase, Cob(I)alamin adenosyltransferase, glycine hydroxymethyltransferase, glutamate formiminotransferase, glycine C-acetyltransferase, Serine C-palmitoyltransferase, Aspartate aminotransferase, Alanine aminotransferase, Cysteine aminotransferase, Glycine aminotransferase, Tyrosine aminotransferase, Leucine aminotransferase, Kynurenine--oxoglutarate aminotransferase, 2,5-diaminovalerate aminotransferase, Histidinol-phosphate aminotransferase, Acetylornithine aminotransferase, Alanine--oxo-acid aminotransferase, Ornithine--oxo-acid aminotransferase, Asparagine--oxo-acid aminotransferase, Glutamine--pyruvate aminotransferase, Succinyldiaminopimelate aminotransferase, Beta-alanine--pyruvate aminotransferase, D-alanine aminotransferase, Diiodotyrosine aminotransferase, Thyroid-hormone aminotransferase, Tryptophan aminotransferase, Pyridoxamine--pyruvate aminotransferase, dTDP-4-amino-4,6-dideoxy-D-glucose aminotransferase, UDP-4-amino-2-acetamido-2,4,6-trideoxyglucose aminotransferase, Glycine--oxaloacetate aminotransferase, L-lysine aminotransferase, (2-aminoethyl)phosphonate--pyruvate aminotransferase, 2-aminoadipate aminotransferase, Branched-chain amino acid aminotransferase, Aminolevulinate aminotransferase, Alanine--glyoxylate aminotransferase, Serine--glyoxylate aminotransferase, Diaminobutyrate--pyruvate aminotransferase, Alanine--oxomalonate aminotransferase, 5-aminovalerate aminotransferase, Dihydroxyphenylalanine aminotransferase, Glutamine--scyllo-inosose aminotransferase, Serine--pyruvate aminotransferase, Phosphoserine aminotransferase, Taurine aminotransferase, Aromatic amino acid transferase, dTDP-4-amino-4,6-dideoxygalactose aminotransferase, Adenosylmethionine--8-amino-7-oxononanoate aminotransferase, Glutamine--phenylpyruvate aminotransferase, N6-acetyl-beta-lysine aminotransferase, Valine--pyruvate aminotransferase, 2-aminohexanoate aminotransferase, D-4-hydroxyphenylglycine aminotransferase, L-seryl-tRNA(Sec) selenium transferase;

dehydratases including propanediol dehydratase, glycerol dehydratase, hydroperoxide dehydratase, myo-inosose-2 dehydratase, L-serine dehydratase, D-serine

dehydratase, Threonine dehydratase, Aminodeoxygluconate dehydratase, Erythro-3-hydroxyaspartate dehydratase;

lyases including methylaspartate ammonia-lyase, ethanolamine ammonia-lyase, mandelonitrile lyase, Ethanolamine-phosphate phospholyase, O-succinylhomoserine (thiol)-lyase, Glucosaminate ammonia-lyase, Carbamoyl-serine ammonia-lyase, Diaminopropionate ammonia-lyase, Cystathionine gamma-lyase, Alliin lyase, S-alkylcysteine lyase, Cystathionine beta-lyase, Cysteine lyase, Methionine gamma-lyase, Cysteine-S-conjugate beta-lyase, Selenocysteine lyase;

synthases including hydroxymethylbilane synthase, glutamate synthase (NADPH), glutamate synthase (ferredoxin)), riboflavin synthase, prostaglandin-D synthase, prostaglandin-E synthase, (S)-stylopine synthase, (S)-cheilanthifoline synthase, berbamunine synthase, salutaridine synthase, (S)-canadine synthase, prostaglandin-I synthase, thromboxane-A synthase, aristolochene synthase, 2-isopropylmalate synthase, Pyrazolylalanine synthase, Tryptophan synthase, Threonine synthase, Cysteine synthase, 1-aminocyclopropane-1-carboxylate synthase, S-carboxymethylcysteine synthase;

phosphatases including aryldialkylphosphatase, diisopropyl-fluorophosphatase, UDP-sugar diphosphatase;

decarboxylases including 5-guanidino-2-oxopentanoate decarboxylase, Aspartate 4-decarboxylase, Valine decarboxylase, Glutamate decarboxylase, Hydroxyglutamate decarboxylase, Ornithine decarboxylase, Lysine decarboxylase, Arginine decarboxylase, Diaminopimelate decarboxylase, Histidine decarboxylase, Aminobenzoate decarboxylase, Tyrosine decarboxylase, Aromatic-L-amino-acid decarboxylase, Sulfinoalanine decarboxylase, Phenylalanine decarboxylase, 2,2-dialkylglycine decarboxylase (pyruvate), Phosphatidylserine decarboxylase;

dehydrogenases including UDP-N-acetylmuramate dehydrogenase, D-lactate dehydrogenase (cytochrome), cellobiose dehydrogenase (quinone), gluconate 2-dehydrogenase, D-2-hydroxy-acid dehydrogenase, pyridoxine 5-dehydrogenase, glucose dehydrogenase (acceptor), glucoside 3-dehydrogenase, malate dehydrogenase (acceptor), D-sorbitol dehydrogenase, retinal dehydrogenase, phenylglyoxylate dehydrogenase (acylating), Succinate dehydrogenase (ubiquinone), succinate dehydrogenase, butyryl-CoA dehydrogenase, acyl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, beta-cyclopiazonate dehydrogenase, isovaleryl-CoA dehydrogenase, 2-methylacyl-CoA dehydrogenase, long-chain acyl-CoA dehydrogenase, D-amino-acid dehydrogenase,

Electron-transferring-flavoprotein dehydrogenase, dimethylglycine dehydrogenase, spermidine dehydrogenase, proline dehydrogenase, NADH dehydrogenase (ubiquinone), NADPH dehydrogenase, NAD(P)H dehydrogenase (quinone), dihydrolipoamide dehydrogenase, 4-cresol dehydrogenase (hydroxylating), dehydrogluconate dehydrogenase, nicotinate dehydrogenase, NADH dehydrogenase, NADPH dehydrogenase (quinone), dimethylmalate dehydrogenase, tartrate dehydrogenase, (R)-aminopropanol dehydrogenase, L-lactate dehydrogenase (cytochrome);

hydroxylases including 3-hydroxyphenylacetate 6-hydroxylase, 4-hydroxybenzoate 1-hydroxylase, isoflavone 3'-hydroxylase, isoflavone 2'-hydroxylase;

reductases including orotate reductase (NADH), 2-enoate reductase, 2-methyl-branched-chain-enoyl-CoA reductase, methylenetetrahydrofolate reductase (NADPH), Cytochrome-b5 reductase, NADPH--ferrihemoprotein reductase, NADPH--cytochrome c2 reductase, glutathione reductase (NADPH), thioredoxin reductase (NADPH), trypanothione reductase, nitrate reductase (NADH), nitrate reductase (NAD(P)H), nitrate reductase (NADPH), nitrite reductase (NAD(P)H), nitrite reductase, 5,10-methylenetetrahydrofolate reductase (FADH), sulfite reductase (NADPH), adenylylsulfate reductase, ferredoxin--NADP(+) reductase, orotate reductase (NADPH), CoA-glutathione reductase (NADPH), aquacobalamin reductase, cob(II)alamin reductase, aquacobalamin reductase (NADPH), cyanocobalamin reductase (NADPH, cyanide-eliminating), hydroxylamine reductase, cucurbitacin delta(23) reductase, benzoyl-CoA reductase; and

monooxygenases including lysine 2-monooxygenase, L-lysine 6-monooxygenase, methylphenyltetrahydropyridine N-monooxygenase, salicylate 1-monooxygenase, 4-hydroxybenzoate 3-monooxygenase, 4-hydroxyphenylacetate 3-monooxygenase, melilotate 3-monooxygenase, imidazoleacetate 4-monooxygenase, orcinol 2-monooxygenase, phenol 2-monooxygenase, dimethylaniline monooxygenase (N-oxide forming), kynurenine 3-monooxygenase, 4-hydroxyphenylacetate 1-monooxygenase, 2,4-dichlorophenol 6-monooxygenase, cyclohexanone monooxygenase, 3-hydroxybenzoate 4-monooxygenase, 3-hydroxybenzoate 6-monooxygenase, 4-aminobenzoate 1-monooxygenase, 4-nitrophenol 2-monooxygenase, albendazole monooxygenase, 4-hydroxybenzoate 3-monooxygenase (NAD(P)H), anthraniloyl-CoA monooxygenase, ketosteroid monooxygenase, benzoyl-CoA 3-monooxygenase, L-lysine 6-monooxygenase (NADPH), squalene monooxygenase, arginine 2-monooxygenase, 2,6-dihydroxypyridine 3-monooxygenase, taxifolin 8-monooxygenase, 4-methoxybenzoate monooxygenase (O-demethylating), latia-luciferin

monooxygenase (demethylating), glyceryl-ether monooxygenase, trans-cinnamate 4-monooxygenase, cholesterol 7-alpha-monooxygenase, 3,9-dihydroxypterocarpan 6A-monooxygenase, leukotriene-B4 20-monooxygenase, methyltetrahydroprotoberberine 14-monooxygenase, tyrosine N-monooxygenase, hydroxyphenylacetonitrile 2-monooxygenase, (-)-limonene 3-monooxygenase, (-)-limonene 6-monooxygenase, (-)-limonene 7-monooxygenase, Protopine 6-monooxygenase, dihydrosanguinarine 10-monooxygenase, dihydrochelirubine 12-monooxygenase, 27-hydroxycholesterol 7-alpha-monooxygenase, unspecific monooxygenase, camphor 5-monooxygenase, steroid 11-beta-monooxygenase, corticosterone 18-monooxygenase, cholesterol monooxygenase (side-chain cleaving), steroid 17-alpha-monooxygenase, steroid 21-monooxygenase, ecdysone 20-monooxygenase, linalool 8-monooxygenase.

Preferred apo-enzymes include apo-oxidases and apo-dehydrogenases, such as apo-glucose oxidase, apo-glucose dehydrogenase, apo-lactate oxidase, and apo-lactate dehydrogenase. An apo-enzyme for sugar is also preferred. The apo-enzyme is inactive and thus does not affect the substrate to which is binds, and thus is a reversible, non-consuming sensor for the substrate.

An anaylte includes any molecule or molecules that can be coupled to an inactive enzyme to form a complex according to the invention, such as substrates for the apo-enzymes listed or incorporated by reference above. Preferably, the analyte is a substrate for an oxidase or a dehydrogenase, such as sugars or esters, preferably glucose or lactate.

A complex is an enzyme that is reversibly coupled to an analyte. "Coupled" refers to a covalent or non-covalent or both a covalent and non-covalent combination, such as the coupling of an enzyme to a substrate.

The inactive enzyme can be labeled using a detectable label, such as a chemical label, *e.g.*, streptavidin and biotin, an enzymatic label, *e.g.*, LacZ and alkaline phosphatase, a radioactive label, *e.g.*, ^{3}H, ^{14}C, ^{335}S, ^{32}P and ^{125}I, a fluorescent label, *e.g.*, GFP, BFP and RFP, or a luminescent label, *e.g.*, luciferase. Preferably, the labeled enzyme is fluorescently labeled at a Cys or Lys residue. In a preferred embodiment, an inactive enzyme, preferably an apo-enzyme, is labeled with 8-anilino-1-naphthalene sulfonic acid (ANS). An apparatus can be used to detect the labeled inactive enzyme and the substrate-bound labeled inactive enzyme. Labels included within the scope of the invention include intrinsic and extrinsic labels. In general, an intrinsic label is a label in which a property of the sample such as mass or charge can affect a second label, e.g., the fluorescence

polarization of a fluorescent label. See Huchzermeier, U.S. Pat. No. 4,476,228. In particular, in one preferred embodiment, an inactive enzyme, preferably an apo-enzyme, changes its intrinsic fluorescence in response to the analyte binding to the enzyme. In another embodiment, the inactive enzyme displays a change in a non-covalently extrinsic fluorophore upon binding with the analyte. In another embodiment, a covalently bound extrinsic probe displays a spectral change. In a further embodiment, the emission, anisotropy, or polarization is dependent on the analyte binding such as lactate binding. In another embodiment, the extent of resonance energy transfer between attached donors and acceptors changes upon analyte binding. A preferred method comprises shining light on a labeled coupled apo-enzyme; determining the intensity of fluorescence; mixing a sample with the labeled coupled apo-enzyme; shining light on the sample and labeled coupled apo-enzyme; and determining the change in intensity of signal, preferably fluorescence. Preferably the labeled apo-enzyme is 8-anilino-1-naphthalene sulfonic acid (ANS) coupled apo-lactate dehydrogenase, ANS coupled apo-lactate oxidase, ANS coupled apo-glucose oxidase, or ANS coupled apo-glucose oxidase.

An assay of the invention can include a heterogeneous or homogenous assay and includes any suitable methodology used for antibodies. "Homogeneous assay" refers to an assay in which the presence and/or concentration of an analyte is determined without requiring the separation of sample fluid from the reaction components. A homogenous assay includes formats in which a detectable signal is only generated upon specific binding of a labeled inactive enzyme to an analyte. As such, homogenous assay formats, the detection occurs without a non-bound labeled enzyme removal step. This broad classification includes many formats known to those skilled in the art.

"Heterogeneous assay" refers to an assay in which a complex is formed, which is removed from the reaction medium before measuring.

In a preferred embodiment, a kit contains instructions for performing the assay, which instructions may be printed on a package insert, packaging or label included in the kit. The printed matter can also be included on receptacles included in the kit, and indicia of sample and reagent volumes can be indicated in the test receptacle. The precise instructions would vary depending upon the analyte to be detected and/or detection method used, but may include instructions for one or more of the following: instructions for dilution of the kit components and/or the sample if necessary, directions for volume or concentration of labeled apo-enzyme used for each assay, volume of sample to add to the

labeled inactive enzyme assay, directions for labeling an inactive enzyme, directions for taking measurement of labeled components, preferred temperature conditions, and timing of component addition and mixing, and use of a standard to calibrate test results.

In one preferred embodiment, the invention includes a method of glucose sensing using an inactive form of glucose oxidase from *Aspergillus niger*. Glucose oxidase was rendered inactive by removal of the FAD cofactor. The resulting apo-glucose oxidase still binds glucose as observed from a decrease in its intrinsic tryptophan fluorescence. 8-Anilino-l-naphthalene sulfonic acid (ANS) was found to bind spontaneously to apo-glucose oxidase as seen from an enhancement of the ANS fluorescence. The steady state intensity of the bound ANS decreased 25% upon binding of glucose, and the mean lifetime of the bound ANS decreased about 40%. These spectral changes occurred with a midpoint from 10 to 20 mM glucose, which is comparable to the KD of hologlucose oxidase. Apo-glucose oxidase can be used as a reversible nonconsuming sensor for glucose, in accordance with the invention.

To prevent glucose oxidation, which prevents the use of the enzyme as a reversible sensor, we removed the FAD cofactor which is required for the reaction. We found that the resulting apo-glucose oxidase still bound glucose with an affinity comparably to the holo enzyme. Importantly, the intrinsic tryptophan fluorescence of apo-glucose oxidase was sensitive to glucose binding. Additionally, apo-glucose oxidase noncovalently bound 8-anilino-l-naphthalene sulfonic acid (ANS). The ANS bound to glucose oxidase displayed decreases in both intensity and lifetime upon addition of glucose. Preferred embodiments of the invention include glucose or other sugar converting enzymes rendered inactive by removal of necessary cofactors. In addition, the invention includes other substrate-converting enzymes rendered inactive by removal of necessary cofactors.

In another preferred embodiment, the present invention includes a protein biosensor for D-glucose based on a thermostable glucose dehydrogenase. The glucose dehydrogenase was non-covalently labeled with 8-anilino-l-naphthalene sulfonic acid (ANS). The ANS-labeled enzyme displayed an approximate 25% decrease in emission intensity upon binding glucose. This decrease can be used to measure the glucose concentration. Our results suggest that enzymes which use glucose as their substrate can be used as reversible and non-consuming glucose sensors in the absence of required cofactors. Moreover, using apo-enzymes for a reversible and non-consuming sensor greatly expands

the range of proteins which can be used as sensors, not only for glucose, but for a wide variety of biochemically relevant analytes, which are also included in the invention.

In the present invention, an enzyme uses glucose as the substrate, but under conditions where no reaction occurs. In one embodiment, a thermophilic and thermostable GD is used that binds glucose, and catalyzes the following reaction:

$$\text{Glucose} + \text{NAD(P)}^+ \rightarrow \text{gluconate} + \text{NAD(P)}^+ + \text{H}^+$$

To prevent the glucose oxidation we used the apo-form of GD, that is, the enzyme without the cofactor which is required for the reaction. Like the apo-GO, the apo-GD still binds glucose with an affinity comparable to the holo-enzyme. Interestingly we found that apo-GD interacts with 8-anilino-l-naphthalene sulfonic acid (ANS) in the presence of organic solvents (e.g. acetone), and that the apo-GD with non-covalently bound ANS displays a decrease in intensity fluorescence upon glucose addition. Our results suggest the use of this enzyme as a biosensor for use in extreme environmental conditions or for extended periods of time.

Based on blood lactate as a clinically valuable diagnostic indicator, one embodiment of the invention is a protein biosensor for L-lactate using lactate dehydrogenase non-covalently labeled with 8-anilino-1-naphthalene sulfonic acid (ANS). The ANS-labeled lactate dehydrogenase displays an approximate 40% decrease in emission intensity upon binding lactate. This decrease is used to measure the lactate concentration. The ANS-labeled lactate dehydrogenase is used in a new easy-to-use apparatus for lactate monitoring which can be used a variety of formats.

As such, this invention includes a method for measuring lactate using lactate dehydrogenase. Lactate dehydrogenase is a tetramer with a molecular weight of 136.700 ± 2,100 daltons. A number of isozymes are known to occur as mixed tetramers of the muscle and heart isozymes. Any of the various isozymes can be used as the sensor.

In the preferred embodiment, beef heart lactate dehydrogenase is non-covalently labeled with 8-anilino-l-naphthalene sulfonic acid (ANS). ANS labeled lactate dehydrogenase displays a decrease in the ANS emission intensity upon binding lactate. This decrease in the ANS fluorescence occurs without consumption of lactate. A change of emission intensity is also expected for other labeled apo-enzymes upon binding to a substrate.

In the preferred embodiment, a simple, easy-to-use apparatus can contain the ANS labeled lactate dehydrogenase and can measure the changes in intensity. Thus, this apparatus can be used to quantify the amount of lactate in a sample of blood, serum, or other bodily fluid. This apparatus can be used at a patients' bedside, doctor's office, at gymnasium, at a sporting event, or other forum. Inactive enzyme sensors, preferably apo-enzyme sensors may be used in a variety of formats including a central clinical lab, a doctor's office testing, home healthcare or a portable device which is worn by a patient.

EXAMPLES

Example 1. Glucose Oxidase

Materials and Methods

Glucose and all other chemicals were of reagent grade and purchased from Sigma. 8-anilino-1-naphthalenesulfonic acid (ANS) and glucose oxidase from *Aspergillus niger* were also obtained from Sigma. Steady state fluorescence measurements were carried out on a SLM AMINCO spectrofluorometer at room temperature, by using quartz cuvettes.

Frequency-domain intensity decay measurements were performed using instrumentation described previously. Lakowicz, J. R., *et al.* (1991) Frequency-domain fluorescence spectroscopy in *Topics in Fluorescence Spectroscopy*, Vol. 1: Techniques (J. R. Lakowicz, Ed.), Plenum Publishing, New York, pp. 293-355. The intensity decay data were analyzed in terms of the multiexponential model.

$$I(t) = \sum_{i,j} \alpha_{i,j} \exp(-t/\tau_i) \qquad (1)$$

In this expression the $\alpha_{i,j}$ values are the initial amplitudes of the component with a decay times (τ_i). The subscript j refers to different concentrations of glucose. The data were analyzed globally with the decay times (τ_j) global for all concentrations of glucose ($\alpha_{i,j}$). The mean lifetime at each glucose concentration was calculated using

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \qquad (2)$$

Preparation of Apo-Glucose Oxidase

A saturated ammonium sulfate solution at 25% was acidified to pH 1.4 with H_2SO_4. 5 mg of glucose oxidase in 0.5 ml solution was added drop-wise with stirring to

5.0 ml of the acidified salt solution at 4°C. The FAD was split off from the enzyme, and the yellow supernatant was removed after centrifugation at 13,000 rpm for 15 min. Swoboda, B. E. P. (1969) *Biochim. Biophys. Acta.* 175, 364-379. The relationship between molecular conformation and the binding of flavin-adenine dinucleotide in glucose oxidase. The precipitate was re-dissolved and neutralized by adding of sodium acetate. The neutralized solution was subjected two more cycles of acidified salt treatment, centrifugation and neutralization. Finally, the protein was washed three times on Centricon tube (Amicon) in 10 mM sodium phosphate, pH 6.8. The apo-glucose oxidase (GO) retained its capacity for reactivation by FAD for several weeks when stored at O°C, and pH 6.8.

In order to exchange the original buffer with 10 mM phosphate buffer, pH 6.8 the enzyme solution was passed through to a PD-10 gel filtration column (Pharmacia). The obtained enzyme was used in our experiments.

Results and Discussion

Our approach to estimate the glucose concentration is based on fluorescence spectral changes which do not require the glucose oxidation. We prepared the FAD-free GO. The apo-enzyme is known to bind the glucose, but it cannot oxide the sugar, Morgan, H. W., *et al.* (1977) *Canadian J. of Microbiol* 23(9), 1109-1117. Figure 1 shows the absorption and emission spectra of apo-GO. The absorbance spectrum shows the characteristic shape of the coenzyme-free proteins, with a maximum of absorbance at 278 nm due to the aromatic amino acid residues. The absence of absorption at wavelengths above 300 nm indicates the FAD has been completely removed. The fluorescence emission spectrum of apo-GO at room temperature upon excitation at 298 nm run displays an emission maximum at 340 nm, which is characteristic of partially shielded tryptophan residues. The addition of 20 mM glucose to the enzyme solution resulted in a quenching of the tryptophanyl fluorescence emission about 18%. This result indicates that the apo-GO is still able to bind glucose. The observed fluorescence quenching may be mainly ascribed to the tryptophanyl residue 426. In fact, as shown by X-ray analysis and molecular dynamics simulations analyses the glucose-binding site of GO is formed by Asp 584, Tyr 515, His 559 and His 516. Moreover, Phe 414, Tip 426 and Asn 514 are in locations where they might form additional contacts to the glucose. Hecht, H. J., *et al.* (1993) *J Mol Biol* 229(l),153-172; Wohlfahrt, G. *et al.* (1999) *Acta. Crystallogr. D. Biol Crystallogr.* 55(5), 967-977; Meyer, M., *et al.* (1998) *J Comp. Aided Molec. Des.* 12(5), 425- 440.

The intrinsic fluorescence from proteins is usually not useful for clinical sensing because of the need for complex or bulky light sources and the presence of numerous proteins in most biological samples. In an attempt to obtain a glucose sensor with longer excitation and emission wavelengths we studied whether ANS would bind to apo-GO. Addition of apo-GO to an ANS solution resulted in an approximate 30-fold increase in the ANS intensity. Importantly, the intensity of the ANS emission was sensitive to glucose, decreasing approximately 25% upon glucose addition (Figure 2). The ANS was not covalently bound to the protein. Addition of glucose resulted in a progressive decrease in the ANS fluorescence intensity. This suggests that the ANS is being displaced into a more polar environment upon binding glucose. The decreased ANS intensity occurred with a glucose-binding constant near 10 mM (Figure 3), which is comparable to the K_D of the holoenzyme. Since the binding affinity has not changed significantly, one can suggest that the binding is still specific for glucose.

Recognizing the favorable attributes of lifetime-based sensing (Szmacinski, H. *et al.* (1994) *supra*; Szmacinski, H. *et al.* (1995) *supra*; Lippitsch, M.E., *supra*) we measured the intensity decay of ANS-labeled apo-GO in the presence of increasing concentrations of glucose (Figure 4). Addition of glucose shifts the frequency responses to higher frequencies, which is due to a decreased ANS lifetime. The shorter lifetimes of ANS apo-GO in the presence of glucose is consistent with our suggestion that glucose displaces the ANS to a more polar environment. The FD data were analyzed globally in terms of the multi-exponential model (Figure 5). The lifetimes were held global at all glucose concentrations. These results show that addition of glucose resulted in a decreased amplitude of the long component with a decay time of 12.3 ns. The decreased amplitude of this component is accompanied by an increased amplitude of the short component with a lifetime of 0.27 ns. These results are consistent with our suggestion that glucose displaces ANS from a hydrophobic to a more polar environment.

Addition of glucose does not affect the amplitude of the middle component with a decay time of 3.4 ns. This suggests that the 3.4 ns component is due to ANS which is bound to the protein but at a site distant from the glucose binding site.

And finally, Figure 6 shows the mean lifetime of ANS apo-GO in the presence of increasing glucose concentrations. The mean lifetime decreases by over 40% upon addition of glucose. These results demonstrate that apo-glucose oxidase, when labeled with suitable fluorophores, can serve as a protein sensor for glucose.

Example 2. Glucose Dehydrogenase

Materials and Methods

Glucose dehydrogenase (GD), ANS and D-glucose were obtained from Sigma. GD was placed in 10 mM sodium phosphate buffer, pH 6.0. This enzyme solution represents the starting material for the fluorescence measurements. For all fluorescence measurements the final concentrations of ANS and GD were 4 μM and 3 μM, respectively. Steady state fluorescence measurements were performed in quartz cuvettes in an ISS spectrofluorometer using magic angle polarizer conditions.

Frequency-domain (FD) measurements were performed using instrumentation described previously. Lakowicz, J. R., *et al.* (1991) In *Topics In Fluorescence Spectroscopy, Volume 1: Techniques*, (Lakowicz, J.R., Ed) Plenum Press, New York, pp. 293-337. For 370 nm excitation, the light source was a frequency doubled pyridine 2 dye laser and the emission observed through a 465 nm interference filter. The FD measurements were also performed using magic angle polarizer conditions. The FD intensity decay data were analyzed by non-linear least squares in terms of the multi-exponential model, also described above.

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \qquad (1)$$

where α_i are the pre-exponential factors associated with the decay time τ_i, with $\sum_i \alpha_i = 1.0$. The mean lifetime is given by

$$\bar{\tau} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} = \sum f_i \tau_i \qquad (2)$$

where fi are the fractional steady state intensities of each lifetime component.

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \qquad (3)$$

The intensity-weighted lifetime is given by

$$\tau = \sum \alpha_i \tau_i \qquad (4)$$

The values of $<\bar{\tau}>$ are thought to be proportional to the quantum yield of the sample.

Polarization Sensing

Polarization sensing provides a method by which a change in intensity is observed as a change in polarization. This polarization is proportional to relative intensities of the sample and the reference. Reference displays a constant intensity and the sample intensity depends on the glucose concentration. The sample (S) and reference (R) sides of the sensor are illuminated with a UV hand lamp. The emission from the sample passes through a vertically oriented polarizer $I^S = I_{II}$, and the emission from the reference passes through a horizontally oriented polar $I^R = I_{\perp}$. The observed polarization P is given by

$$P = \frac{I^s - I^r}{I^s + I^r} \qquad (5)$$

We assumed that the initial ratio of the sample to the reference (k) is given by

$$k = \frac{I_0^s}{I_0^r} \qquad (6)$$

The initial polarization of the sample will be P_0,

$$P_0 = \frac{I_0^s - I_0^r}{I_0^s + I_0^r} = \frac{k-1}{k+1} \qquad (7)$$

If in response to analyte the sample intensity will change n-times, it is possible to calculate

$$P = \frac{nk-1}{nk+1} \qquad (8)$$

and the observed changes in polarization, ΔP, will be

$$\Delta P = P_0 - P = \frac{k-1}{k+1} - \frac{nk-1}{nk+1} \qquad (9)$$

Equation (9) describes the dependence of observed changes in polarization (ΔP) on the values of n and k. It is interesting to consider values of k needed to obtain the maximum change of ΔP for different values of n.

In Figure 7 are shown the simulations of the dependence of ΔP as a function of k for different values of n. From this theoretical prediction, it is possible to optimize the initial

experimental conditions in order to get the widest change of polarization upon analyte addition (e.g. n=0.1; k~3). For instance, the intensity of our glucose-sensing protein decreases by a factor n=0.7 upon saturation with glucose. In this case the maximum change in polarization is obtained when the initial intensity ratio is near k=1.3, that is, the intensity of the reference is about 75% that of the sample.

Results

ANS is known to be a polarity-sensitive fluorophore which displays an increased quantum yield in low polarity environments. Weber, G. (1951). *Biochem J.* 51:155-167; Slavik, J. (1982). *Biochim. Biophys. Acta* 694:1-25. Additionally, ANS frequently binds to proteins with an increase in intensity. We examined the effects of GD on the emission intensity of ANS. A moderate enhancement was found but the ANS intensity remained low compared to other ANS-protein complexes. Also, addition of glucose to this GD-ANS complex did not change upon addition of glucose.

GD is a thermophilic protein and can be expected to be rigid under mesophilic conditions. We knew that thermophilic proteins often display increased activity at higher temperatures or the presence of non-polar solvents (D'Auria, S., *et al.* (1999) *Biophys. Chem* 81, 23-31; D'Auria, S., *et al.* (1999) *J. Biochem.* 126, 545-552) which are conditions expected to increase the protein dynamics. Addition of acetone to the solution containing ANS and GD resulted in a dramatic increase in the ANS intensity, (Figure 8), as well as in a blue-shift of the emission maximum. Addition of similar amounts of acetone to ANS in the absence of the protein produced modest fluorescence increase. Hence the increase in the ANS intensity reflects a change in the local protein environment which is due to acetone.

To be useful as a glucose sensor, the ANS-labeled GD must display usefully large spectral changes in the presence of glucose. Addition of glucose to ANS-GD in the presence of 3% acetone resulted in an approximate 25% decrease in intensity (Figure 9). This seemed to be the optimal acetone concentration because smaller spectral changes were seen at lower and higher acetone concentrations (Figure 10). Apparently at higher acetone concentrations the ANS is already in an environment which results in much of the possible increase in quantum yield. At lower acetone concentrations the environment surrounding the ANS changes in response to glucose in a manner which increases the ANS intensity.

In previous reports we described the value of fluorescence lifetimes as a basis for chemical sensing. Lakowicz, J.R., *et al.* (1993). *Sensors and Actuators* B 11, 133-143;

Szmacinski, H., *et al.* (1994) *Topics in Fluorescence Spectroscopy* 4:295-334. Hence we questioned whether the glucose-dependent decrease in intensity would be accompanied by a similar change in the ANS decay time. The frequency-domain intensity decay of ANS-GD are shown in Figure 11. Glucose induces a modest shift in the response to higher frequencies, which indicate a decrease in the mean decay time. In the presence and absence of glucose the multi-exponential analysis (Table 1) indicates that the decay is dominated by a sub-nanosecond component whose contribution is increased by glucose. However the changes in the intensity decay, or equivalent the phase and modulation, are not adequate for lifetime-based sensing.

In the preceding discussion we interpreted the results in terms of a change in the protein environment caused by glucose. However, it is also possible that the changes are due to a difference in the amount of protein-bound ANS due to glucose.

In previous reports we described the use of polarization sensing for systems which display changes in intensity, but not lifetime, in response to analytes. Lakowicz, J.R., *et al.* (1999) *Anal. Chem.* 71:1241-1251; Gryczynski, Z., *et al.* (2000) *Optional Engineering*, (in press). Because the intensity changes of ANS-GD in response to glucose are modest, it is important to carefully select the best conditions.

Figure 12 shows the emission polarized spectra of ANS-GD at various concentrations of glucose. The polarization decreases at higher glucose concentrations because the emission from this solution is observed through the horizontal polarizer. Moreover, the change in polarization is larger at shorter wavelengths, and this is due to the differences in the emission spectra of reference (ANS in buffer) and sample (ANS+GD). The wavelength dependent changes in polarization were used to create a calibration curve for glucose (Figure 13). This curve shows that the present ANS-GD system can yield glucose concentrations accurate to about ±2.5 mM, at a glucose concentration near 20 mM.

Discussion

The results described above represent our first attempt to use GD as a glucose sensor. We feel the performance of GD with noncovalently bound ANS is marginal for a glucose sensor. A larger glucose-dependent spectral change would increase the accuracy of the glucose measurements. The non-covalent binding of ANS and GD is a disadvantage because changes in the ANS or GD concentration might alter the glucose calibration curve. And finally, the need for acetone to increase the response to glucose is problematic because it is unlikely that acetone would be present in a clinically useful glucose sensor.

In spite of these difficulties we feel the ANS-GD system demonstrates a useful approach to sensing. Our results suggest that the enzymes which use glucose as their substrate can be used as reversible and non-consuming glucose sensors in the absence of required co-factors. Using inactive apo-enzymes for a reversible sensor greatly expands the range of proteins which can be used as sensors, not only for glucose, but for a wide variety of biochemically relevant analytes, which are included in the scope of the invention. Hence one is no longer limited to using signaling proteins which bind the analyte without chemical reaction. The need for acetone may be eliminated by selecting proteins which are less thermophilic. The proteins can be engineered for covalent labeling by insertion of cysteine residues at appropriate locations in the sequence. The glucose induced spectral changes may be larger with other polarity sensitive probes or by the use of RET between two fluorophores on the protein. In summary, apo-enzymes are found to be a valuable source of protein sensors.

Table 1

Multi-exponential intensity decay of ANS-labeled GD in the absence and presence of glucose

[Glucose]	$\bar{\tau}$ (ns)[a]	$\langle\tau\rangle$(ns)[b]	α_i	f_i	τ_i(ns)	χ_R^2
0.0	0.46	2.00	0.96	0.62	0.28	0.8
			0.04	0.38	4.86	
70Mm	0.39	1.69	0.97	0.66	0.27	0.7
			0.03	0.34	4.40	

[a] $\bar{\tau} = \sum_i f_i \tau_i$

[b] $\langle\tau\rangle = \sum_i \alpha_i \tau_i$

The uncertainties in the phase and modulation were taken as $\delta\varphi = 0.30$ and $\delta m = 0.007$, respectively.

Example 3. Lactate Dehydrogenase

In this example, beef heart lactate dehydrogenase, ANS and L-lactate were obtained from Sigma. Lactate dehydrogenase was extensively dialyzed against mM sodium phosphate buffer, pH 6.0 at 4°C. After dialysis the enzyme solution was centrifuged at 12,000 rpm for 30 min at 4°C and the supernatant was recovered. The supernatant was filtered by utilizing an inorganic membrane filter, Anotop 10 (Whatman). The obtained enzyme solution represents the starting material for the fluorescence measurements. For all

fluorescence measurements the final concentrations of ANS and lactate dehydrogenase were 4 μM and 3 μM, respectively. Steady state fluorescence measurements were performed in quartz cuvettes in an ISS spectrofluorometer using magic angle polarizer conditions.

Frequency-domain (FD) measurements were performed using instrumentation described previously. Lakowicz, J. R., *et al.* (1991). Frequency-domain fluorescence spectroscopy in *Topics in Fluorescence Spectroscopy, Volume 1: Techniques,* (Lakowicz, J. R., Ed), Plenum Press, New York, pp. 293-337; Laczko, G., *et al.* (1990) *Rev. Sci. Instrum.* 61:2331-2337.

For excitation at 295 nm the light source was a frequency-doubled cavity dumped R6G dye laser, and the emission observed through 340 nm interference filter. For 370 nm excitation the light source was a Pyridene 2 dye laser and the emission observed through a 465 nm interference filter. The FD measurements were a performed using magic angle polarizer conditions.

As discussed above, the FD intensity decay data were analyzed by non-linear least squares in terms of multi-exponential model

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \tag{1}$$

where α_i are the pre-exponential factors associated with the decay time τ_i, with $\Sigma_i \alpha_i = 1.0$. The mean lifetime is given by

$$\bar{\tau} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} = \sum f_i \tau_i \tag{2}$$

where f_i are the fractional steady state intensifies of each lifetime

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{3}$$

The intensity-weighted lifetime is given by

$$<\bar{\tau}> = \sum_i \alpha_i \tau_i \tag{4}$$

The values of $<\bar{\tau}>$ are thought to be proportional to the quantum yield of the sample.

Figure 14 shows the intrinsic tryptophan emission of ANS-coupled lactate dehydrogenase. Addition of micromolar concentrations of lactate resulted in an approximate 30% decrease in the tryptophan intensity, consistent with an earlier report on

the C_4 isozyme of lactate dehydrogenase. Gupta, G. S., *et al.* (1997) *Indian J. of Biochemistry & Biophysics* 34:307-312. An increase of lactate concentration over 200 μM does not introduce further changes in fluorescence intensity. Thus, the enzyme is mostly saturated by 200 μM lactate.

As shown in Figure 15 and Table 2, lactate binding did not significantly change nor affect the mean lifetime, intensity-weighted lifetime or intensity decay of lactate dehydrogenase. The frequency-domain intensity decays are shown in Figure 15, and the multi-exponential analysis of these data are given in Table 2. There was no significant change in due to lactate binding. Given the modest change in the emission intensity, and the difficulty of using intrinsic protein fluorescence in a clinical setting, the intrinsic emission of lactate dehydrogenase is not useful for lactate sensing. It is apparent from the above that one could test other apo-proteins for use as non-consuming sensors. This testing could be accomplished by isolating the purified protein and removing cofactors or prosthetic groups which are necessary for the activity and/or reaction. One then examines the intrinsic fluorescence of the protein, or the fluorescence of a protein labeled with another fluorophore. If significant changes are observed in intensity, wavelength ratio intensity polarization, anisotropy energy transfer or lifetime, than the apo-enzyme is a candidate for a sensor.

A clinically useful sensor requires that the wavelength be long enough to allow the use of simple excitation sources. Within the past several years UV output near 370 nm has become available from light emitting diodes (LED) (Sipior, J., *et al.* (1995) *Anal. Biochem.* 20:309-318; Szmacinski, H., *et al.* (1999) *Applied Spectroscopy* 54:138__; Sipior, J., *et al.* (1997) *Rev. Sci. Instrum.* 68(7):2670-2666), and laser diodes as short as 399 nm have been reported. Landgraf, S., *et al.* (1998) *J. Inf. Recording* 24:141-148; Someya, T., *et al.* (1999) *Science* 285:1905-1906. LEDs are also known to be useful for ns lifetime measurements because of the capability of high frequency modulation. Szmacinski, H., *supra*; Sipior J., *supra*, Fantini, S., et al. (1994) *Applied Optics* 33:52045213; Sipior, J., et al. (1996) *Rev. Sci. Instrum.* 67(11):3795-3798.

In the preferred embodiment, lactate dehydrogenase is labeled with the fluorophore, 8-anilino-1-naphthalene sulfonic acid, which is suitable for LED excitation. The emission intensity of ANS solution with lactate dehydrogenase was about 30-fold higher and displayed a blue shift from 525 to 465 nm. ANS concentrations significantly higher than the lactate dehydrogenase concentration did not appear to bind to lactate dehydrogenase.

Others have reported that tetrameric lactate dehydrogenase binds 4 to 6 ANS molecules, and further addition of ANS does not result in further ANS binding. Kube, D. *et al.* (1987) *Biokhim* 52(2):179-187; Ivanov, M.V. *et al.* (1984) *Biochim. Biophys. Acta* 789(2):216-223. Importantly, the emission intensity of the lactate dehydrogenase-bound ANS was sensitive to lactate, and decreased about 40% upon lactate binding (see Figures 16 and 17). The mean lifetime of ANS decreased upon binding of lactate (see Figure 18 and Table 3). However, these lifetime changes were rather modest and were judged to be too small for lifetime-based sensing. Szmacinski, H., *et al.* (1994) Lifetime-Based Sensing in Topics in *Fluorescence Spectroscopy, Volume 4: Probe Design and Chemical Sensing,* (Lakowicz, J. R., Ed.) Plenum Press, New York, Chapter 10 pp. 295-334.

Polarization sensing is accomplished by constructing a sensor such that a stable intensity reference is observed through one polarizer and the sample is observed through a second orthogonal polarizer. One such configuration is shown in Figure 19. In this case the reference is a ANS-lactate dehydrogenase solution in the absence of lactate, which can be expected to display similar temperature, time or illumination-dependent changes as the sample. This reference is observed through a vertically oriented polarizer. The sample contains ANS-lactate dehydrogenase and various concentrations of lactate, and is observed through a horizontally oriented polarizer. The emission from both sides of the sensor is then observed through an analyzer polarizer. The analyzer polarizer is rotated until the emission from both sides is equalized, which can be measured visually (Gryczynski, I., *et al.* (1999) *Anal. Chemistry* 71:1241-1251) or with a simple photocell or photodiode circuit shown in Figure 19. Gryczynski, Z., *et al.* (2000) *Optical Engineering* in press. In this case the analyzer polarizer is rotated until the voltage across the differential electronics (Watson Bridge) is zero. The angle of polarizer rotation can then be used to determine the lactate concentration. This angle is called the "compensation angle."

In the previous publications we calculated the change in compensation angle expected for various changes in sample intensity. The simulations in Figure 19 are for intensity changes comparably to those observed for ANS-LDH. The lower panel of the Figure 19 shows the compensation angle dependence, from $\Delta\alpha$ different initial ratio of reference to sample fluorescence intensity, $k = I_R/I_S$. Series of curves are plotted for different values of n which represents expected total intensity change of sensing fluorophore in response to analyte. These simulations show that a 40% change in intensity is only expected to result in a modest 6° change in the compensation angle.

The apparatus in Figure 19 was used with a dual photodiode detector to measure lactate concentrations (Figure 20). The intensity change induced by analyte (lactate) results in a change of the compensation angle, $\Delta\alpha$, which is related to the concentration of analyte. Gryczynski, I. *supra*; Gryczynski, Z., *supra*. As predicted from Figure 19, the total intensity change ~40% (n-1.7) the change in compensation angle was about 6° for the entire range of lactate concentrations. While the range seems small, the compensation angles are readily measured to about 0.1 degrees, so that a 6° change corresponds to an accuracy of 2% in the lactate concentration. Except for the UV handlamp light source, the device shown in Figure 19 was battery powered and could be easily designed as a portable instrument for bedside use.

The simulations in Figure 19 are for intensity changes comparably to those observed for ANS-lactate dehydrogenase. The lower panel of the Figure 19 shows the compensation angle dependence, $\Delta\alpha$ from different initial ratio of reference to sample fluorescence intensity, $k = I_R/I_S$. Series of curves are plotted for different values of n which represents expected total intensity change of sensing fluorophore in response to analyte. These simulations show that a 40% change in intensity is only expected to result in a modest 6° change in the compensation angle.

In this preferred embodiment, the beef heart lactate dehydrogenase was only moderately stable at room temperature and had to be used within several days following removal from the ammonium sulfate solution.

In alternative embodiments, one can improve the stability of lactate dehydrogenase in a variety of manners. One manner is by immobilization of the lactate dehydrogenase into a matrix which often stabilizes proteins. Kotorman, M., *et al.* (1986). *Biotechnol. Affl. Biochem. 8:53-69.* An alternative embodiment may involve using lactate dehydrogenase from thermophilic organisms or mesophilic organisms.

Another alternative embodiment may include modifying lactate dehydrogenase to obtain a larger spectral change, preferably with a change in lifetime or a useful spectral shift. Site directed mutagenesis of lactate dehydrogenase could result in lactate dehydrogenase with more stability and larger and more useful spectra changes.

An apparatus which uses the ANS coupled lactate dehydrogenase to measure lactate in blood, serum, or other bodily fluids is possible. This apparatus is easy-to-use and provides quick results.

This apparatus uses, in its preferred embodiment, polarization sensing to quantify the amount of lactate present in the sample. Other types of sensors can be used which can use various fluorescence measurements including emission intensity, emission maxima, spectral shift, wavelength-ratio, energy transfer and lifetime. Light sources can include laser diodes, LEDs, and similar devices.

In the apparatus shown in the top panel of Figure 19, the sample (S) and reference (R) sides of the sensor are illuminated with a UV hand lamp. The emission from the reference passes through a vertically oriented polarizer, and the emission from the sample passes through a horizontally oriented polarizer. The emission from S and R is observed through an analyzer polarizer (AP) using a dual photocell.

$$I^{V} = I_{//}^{R} \cos^2\alpha \qquad (10)$$

The emissions passing through analyzer polarizer from the vertical and horizontal sides of the sensor are given by

$$I^{H} = I_{\perp}^{S} \sin^2\alpha \qquad (11)$$

where α is the angular displacement of the analyzer polarizer from the vertical position. The analyzer is rotated until the intensity from reference and sample are equal. For this condition one has

$$I_{//}^{R}\cos^2\alpha = I_{\perp}^{S}\sin^2\alpha \qquad (12)$$

and

$$\tan^2\alpha = \frac{I_{//}^{R}}{I_{\perp}^{S}} \qquad (13)$$

A change in the sample intensity induced by the analyte results in changes of the analyzer polarizer angle needed to equalize the intensities. This difference is shown as the compensation angle, $\Delta\alpha$.

$$\Delta\alpha = \alpha_0 - \alpha \qquad (14)$$

In this expression α_o, refers to the rotation angle of analyzer polarizer (AP) needed to equalize the intensities in the absence of analyte and α is the angle of AP needed to equalize the reference and sample intensities in the presence of given analyte concentration. Gryczynski, I. *supra*; Gryczynski, Z., *supra*.

In order to obtain the maximum change in compensation angle, $\Delta\alpha$, one should consider the initial intensity ratio of reference to sample fluorescence intensities $k = I^R/I^S$ where I^R and I^S are the total intensities. Suppose the intensity of the sensing fluorophore

decrease n-times in response to analyte. Then the change in compensation angle is given by

$$\Delta\alpha = a\tan\sqrt{\frac{I^R}{I^s}} - a\tan\sqrt{\frac{nI^R}{I^s}} \qquad (15)$$

In the preferred embodiment, the ANS-coupled lactate dehydrogenase does not consume nor alter the lactate in the sample. While this aspect of not changing the lactate is preferable, it is possible that the fluorescent molecule coupled enzyme alters or changes the lactate in the sample.

Figure 19 shows simulations of the expected changes in compensation angle $\Delta\alpha$ as a function of initial ratio of the reference to sample fluorescence, k, for different values of n.

In alternative embodiments, one can use lactate oxidase coupled to a fluorescent molecule instead of lactate dehydrogenase. Furthermore, the lactate oxidase or the lactate dehydrogenase can be derived from thermophilic or mesophilic organisms. In addition, one can mutate specific amino acids of lactate dehydrogenase or lactate oxidase from non-thermophilic or non-mesophilic organisms to generate enzymes that are stable at the temperatures needed for this invention. It is preferable to have the sensing enzyme active at both room temperature and at temperatures higher than 37 degree Celsius. In an alternative embodiment, one can immobilize the enzyme to increase its ability to withstand temperatures higher than 37 degrees Celsius.

Table 2
Multi-exponential intensity decay of the intrinsic emission of LDH in the absence and presence of lactate

[Lactate]	$\langle\tau\rangle$(ns)[a]	$\langle\bar{\tau}\rangle$(ns)[b]	α_i	f_i	τ_i(ns)	χ_R^2
0.0	3.21	5.54	0.319	0.057	0.60	0.8[c]
			0.399	0.312	2.60	
			0.282	0.631	7.47	
240 μM	3.21	5.33	0.339	0.066	0.63	0.6
			0.363	0.280	2.48	
			0.298	0.653	7.05	

a $\langle\tau\rangle = \Sigma_i\alpha_i\tau_i$

b $\bar{\tau} = \Sigma_i f_i\tau_i$

c The uncertainties in the phase and modulation were taken as $\delta\varphi = 0.30$ and $\delta m = 0.007$, respectively.

Table 3
Multi-exponential intensity decay of ANS-labeled LDH in the absence and presence of lactate

[Lactate]	$\langle\tau\rangle$(ns)[a]	$\langle\bar{\tau}\rangle$(ns)[b]	α_i	f_i	τ_i(ns)	χ_R^2
0.0	9.73	14.8	0.217	0.017	0.78	0.8[c]
			0.290	0.154	5.16	
			0.493	0.829	16.9	
240 μM	9.01	14.4	0.265	0.022	0.77	0.7
			0.324	0.208	5.89	
			0.412	0.770	17.1	

a $\langle\tau\rangle = \Sigma_i\alpha_i\tau_i$

b $\bar{\tau} = \Sigma_i f_i\tau_i$

c The uncertainties in the phase and modulation were taken as $\delta\varphi = 0.30$ and $\delta m = 0.007$, respectively.

The invention having been fully described, other embodiments and modifications of the invention may be apparent to those of ordinary skill in the art. All such embodiments and modifications are within the scope of the invention, which is defined by the appended claims.

We claim:

1. A method for assaying a sample suspected of containing an analyte comprising:

(a) contacting the sample with an inactive enzyme to form a complex; wherein the analyte is not consumed in the assay, and wherein the enzyme is coupled to the analyte during the contacting step; and

(b) measuring the amount or presence of the analyte coupled to the enzyme.

2. The method of claim 1 wherein the inactive enzyme comprises a label.

3. The method of claim 2 wherein the label is an intrinsic label or an extrinsic label.

4. The method of claim 3 wherein the label is selected from the group consisting of a fluorescent label, a luminescent label, an enzyme label, a radioactive label, and a chemical label.

5. The method of claim 4 wherein the label is a fluorescent label.

6. The method of claim 5 wherein the label is 8-anilino-1-naphthalene sulfonic acid (ANS).

7. The method of claim 2 wherein in step (b) the coupled analyte is measured by emission maxima, emission intensity, spectral shift, energy transfer, anisotropy, polarization, lifetime or wavelength ratio.

8. The method of claim 1 wherein the inactive enzyme is a mutated enzyme, an apo-enzyme, or an inhibited enzyme.

9. The method of claim 8 wherein the inactive enzyme is an apo-enzyme.

10. The method of claim 1 wherein the analyte is a substrate for an oxidase or a dehydrogenase enzyme.

11. The method of claim 1 wherein the analyte is a sugar.

12. The method of claim 11 wherein the analyte is glucose.

13. The method of claim 12 wherein the inactive enzyme is apo-glucose oxidase or apo-glucose dehydrogenase.

14. The method of claim 1 wherein the analyte is lactate.

15. The method of claim 14 wherein the inactive enzyme is apo-lactate dehydrogenase or apo-lactate oxidase.

16. The method of claim 1, wherein the enzyme is produced from a thermophilic organism.

17. The method of claim 1 wherein the assay is a homogenous assay.

18. The method of claim 1 wherein the assay is a heterogeneous assay.

19. The method as in claim 1, wherein the sample is whole blood, serum or plasma.

20. The method of claim 1

wherein the analyte is glucose and the inactive enzyme is apo-glucose oxidase or apo-glucose dehydrogenase labeled with ANS, or the analyte is lactate and the inactive enzyme is apo-lactate dehydrogenase or apo-lactate oxidase labeled with ANS,

wherein the inactive enzyme is produced from a thermophilic organism,

wherein the assay is a homogenous assay,

wherein the sample is whole blood, serum or plasma, and

wherein the amount or presence of the coupled analyte is measured by fluorescence.

21. A kit for assaying a sample suspected of containing an analyte, the kit comprising a composition having an inactive enzyme

wherein the inactive enzyme does not consume the analyte upon assaying.

22. The kit of claim 21 wherein the inactive enzyme comprises a label.

23. The kit of claim 22 wherein the label is an intrinsic label or an extrinsic label.

24. The kit of claim 23 wherein the label is selected from the group consisting of a fluorescent label, a luminescent label, an enzyme label, a radioactive label, and a chemical label.

25. The kit of claim 24 wherein the label is a fluorescent label.

26. The kit of claim 25 wherein the label is ANS.

27. The kit of claim 21 wherein the inactive enzyme is a mutated enzyme, an apo-enzyme, or a covalently inhibited enzyme.

28. The kit of claim 27 wherein the inactive enzyme is an apo-enzyme.

29. The kit of claim 28 wherein the apo-enzyme is apo-glucose oxidase, apo-glucose dehydrogenase, apo-lactate dehydrogenase, or apo-lactate oxidase.

30. The kit of claim 1, wherein the enzyme is produced from a thermophilic organism.

31. The kit of claim 21, further comprising instructions for assaying the sample.

32. The kit of claim 21 further comprising instructions for assaying a sample suspected of containing the analyte,

wherein the inactive enzyme is labeled with ANS and is an apo-glucose oxidase, apo-glucose dehydrogenase, apo-lactate dehydrogenase or apo-lactate oxidase.

33. An assay for an analyte in a sample suspected of containing an analyte comprising:

the sample and an inactive enzyme capable of forming a complex,

wherein the analyte reversibly couples to the inactive enzyme, and wherein the inactive enzyme does not consume the analyte.

34. The assay of claim 33 wherein the inactive enzyme comprises a label.

35. The assay of claim 34 wherein the label is an intrinsic label or an extrinsic label.

36. The assay of claim 35 wherein the label is selected from the group consisting of a fluorescent label, a luminescent label, an enzyme label, a radioactive label, and a chemical label.

37. The assay of claim 36 wherein the label is a fluorescent label.

38. The assay of claim 38 wherein the label is ANS.

39. The assay of claim 33 wherein the analyte is a substrate for an oxidase or a dehydrogenase enzyme.

40. The assay of claim 33 wherein the analyte is a sugar.

41. The assay of claim 40 wherein the analyte is glucose.

42. The assay of claim 33 wherein the inactive enzyme is a mutated enzyme, an apo-enzyme, or an inhibited enzyme.

43. The assay of claim 42 wherein the inactive enzyme is an apo-enzyme

44. The assay of claim 43 wherein the apo-enzyme is an apo-glucose oxidase or apo-glucose dehydrogenase.

45. The assay of claim 33 wherein the analyte is lactate.

46. The assay of claim 45 wherein the apo-enzyme is apo-lactate dehydrogenase or apo-lactate oxidase.

47. The assay of claim 1, wherein the enzyme is produced from a thermophilic organism.

48. The assay of claim 33, wherein the sample is whole blood, serum or plasma.

49. The assay of claim 33 wherein the assay is a homogenous assay.

50. The assay of claim 33 wherein the assay is a heterogeneous assay.

51. The assay of claim 25

wherein the analyte is glucose and the inactive enzyme is apo-glucose dehydrogenase or apo-glucose oxidase labeled with ANS, or the analyte is lactate and the inactive enzyme is apo-lactate dehydrogenase or apo-lactate oxidase labeled with ANS,

wherein the inactive enzyme is produced from a thermophilic organism,

wherein the assay is a homogenous assay, and

wherein the sample is whole blood, plasma or serum.

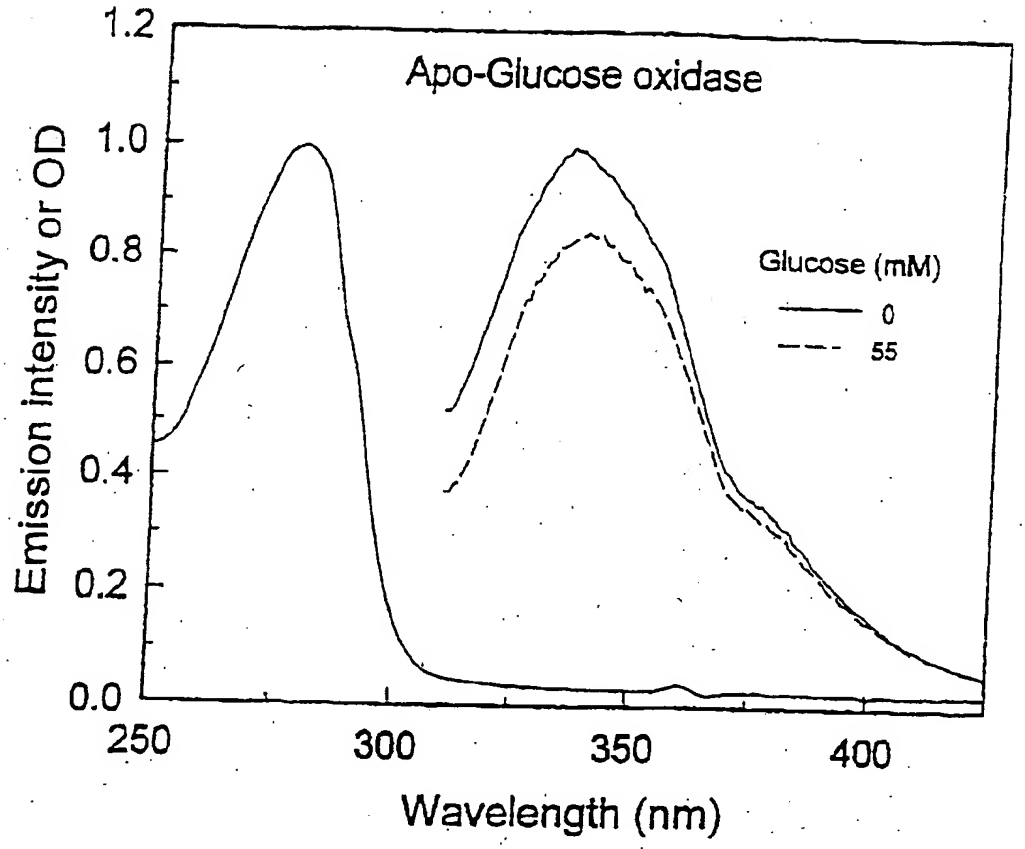


Figure 1. Absorption and emission spectra of apo-glucose oxidase. Excitation at 298 nm. The protein concentration was 0.05 mg/ml.

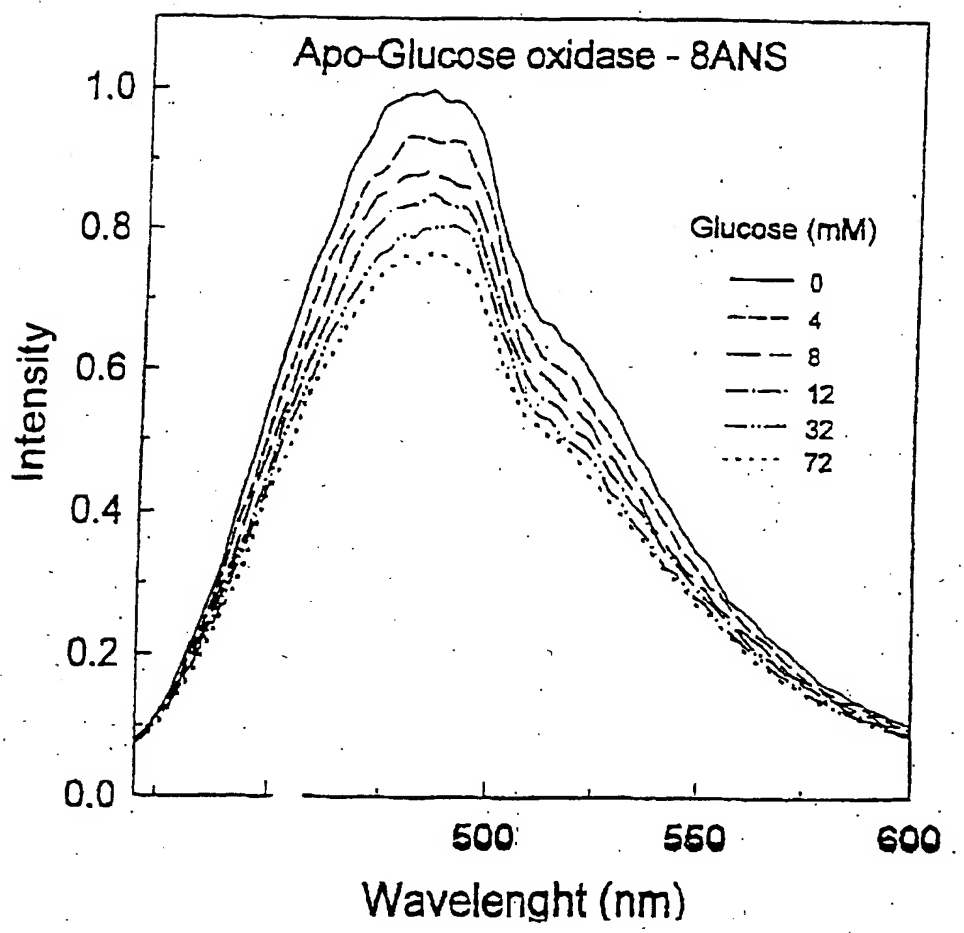


Figure 2. Emission spectra 5 μM 1,8-ANS in the presence of 3 μM apo-glucose oxidase. Excitation at 325 nm.

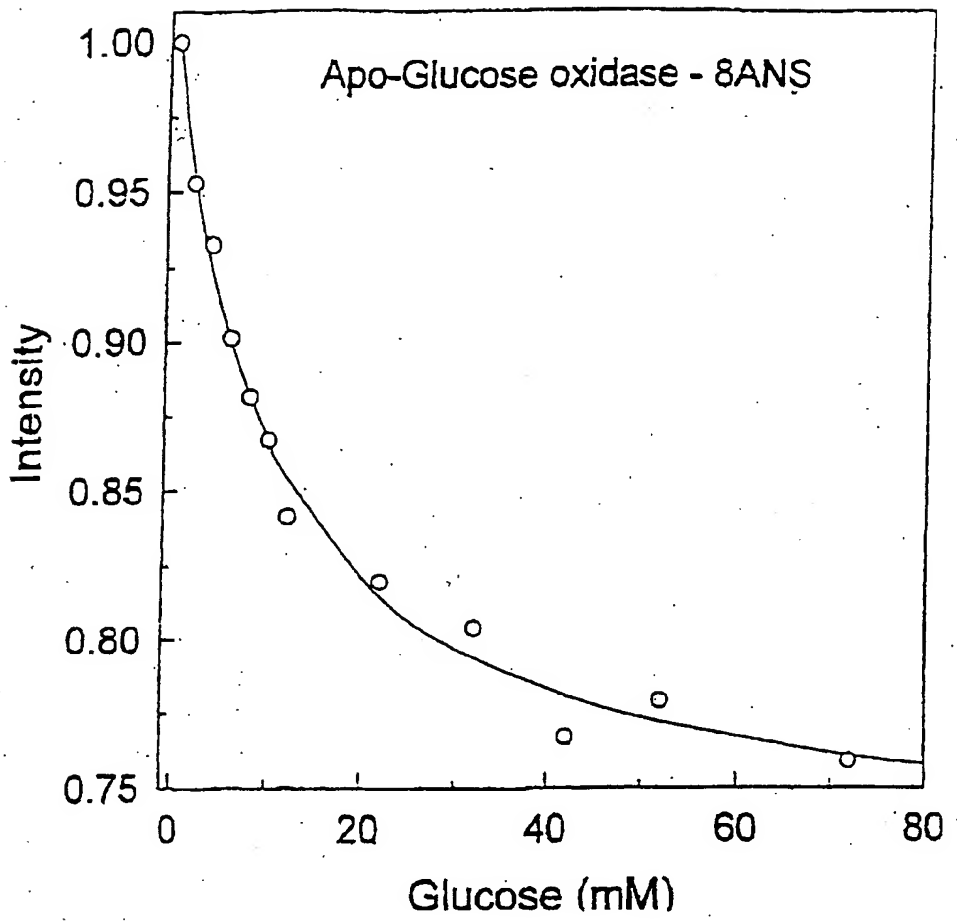


Figure 3. Glucose-dependent emission intensity of 1,8-ANS bound to apo-glucose oxidase. Excitation at 325 nm, emission at 480 nm.

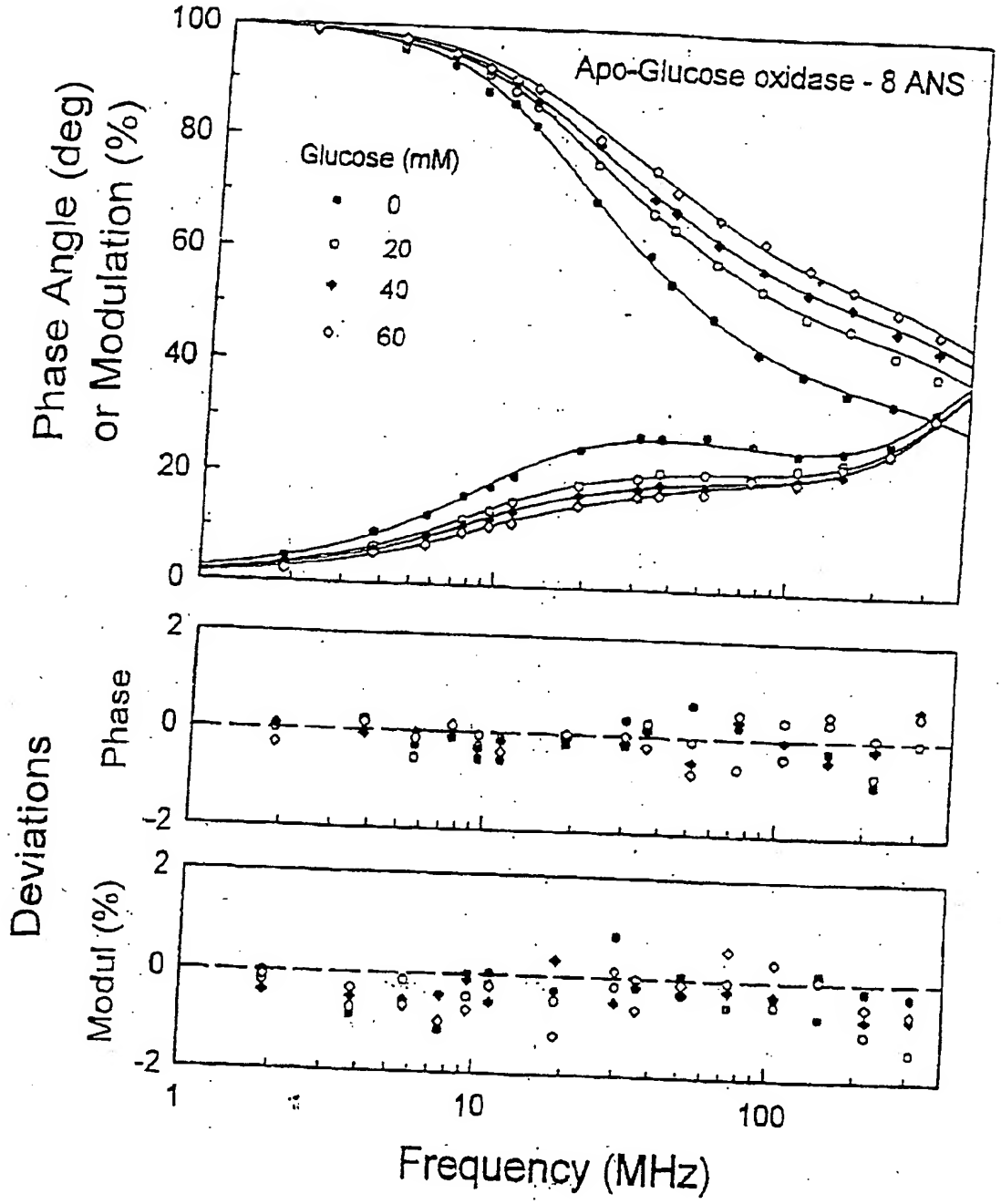


Figure 4. Frequency-domain intensity decays of 1,8-ANS-apo-glucooxidase in the presence of increasing concentrations of glucose.

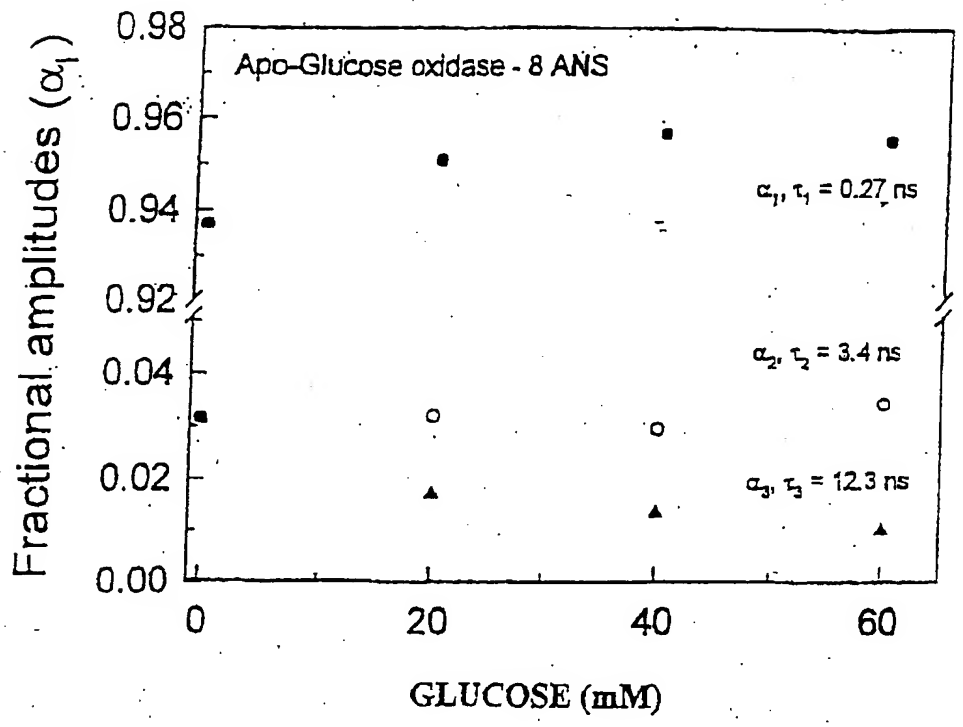


Figure 5. Glucose-dependent lifetimes and pre-exponential factors from the lifetime-global analysis.

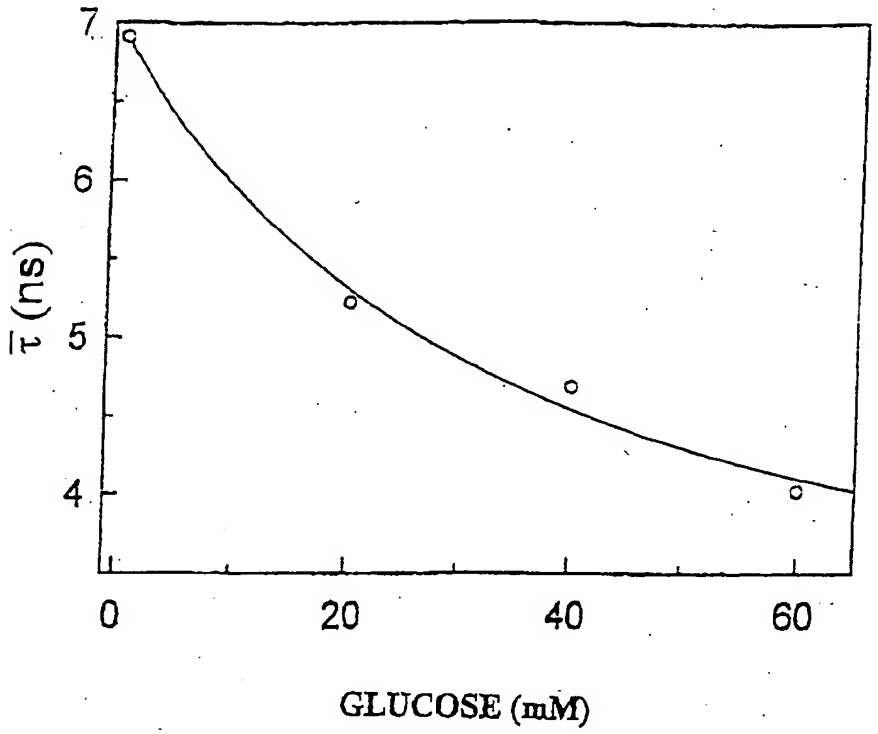


Figure 6. Effect of glucose on the mean decay time of 1,8-ANS labeled apo-glucose oxidase.

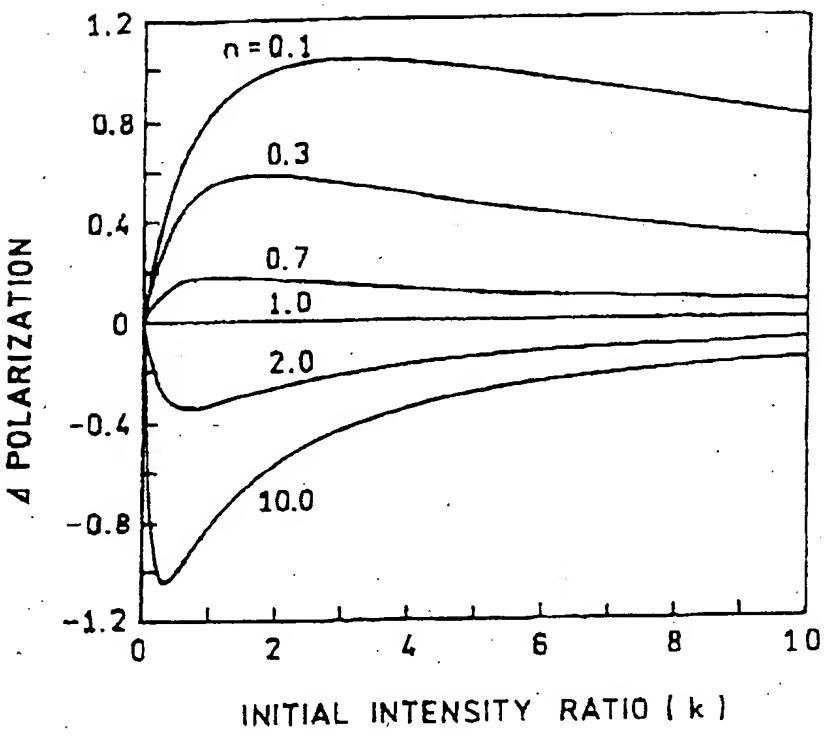


Figure 7. Polarization sensing. Simulations of the expected changes in polarization for different values of k. For details see Materials and Methods section.

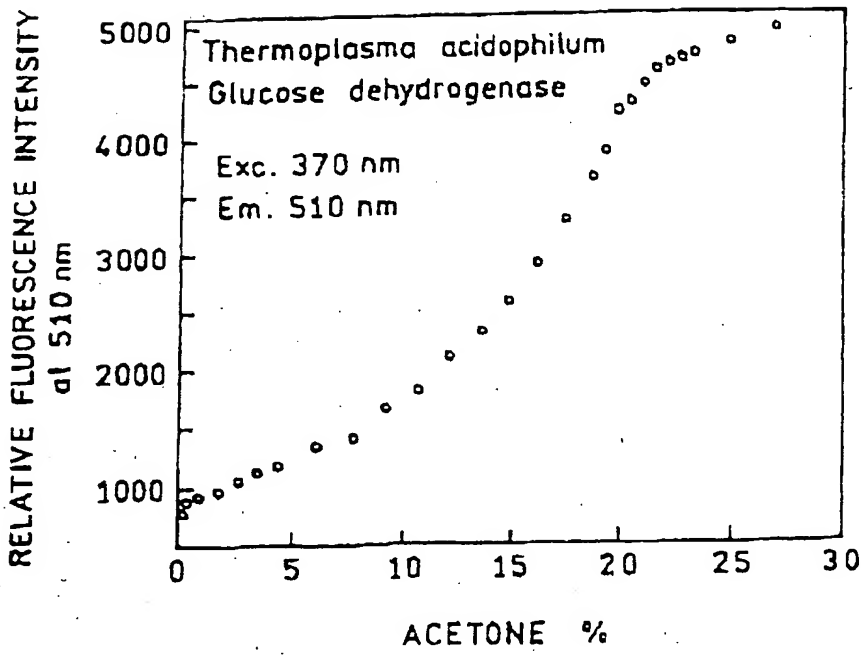


Figure 8. ANS-labeled GD fluorescence intensity in the presence of different concentrations of acetone. [GD] = 3 μM. [ANS] = 4 μM. The excitation was at 370 nm, and the emission was monitored at 510 nm.

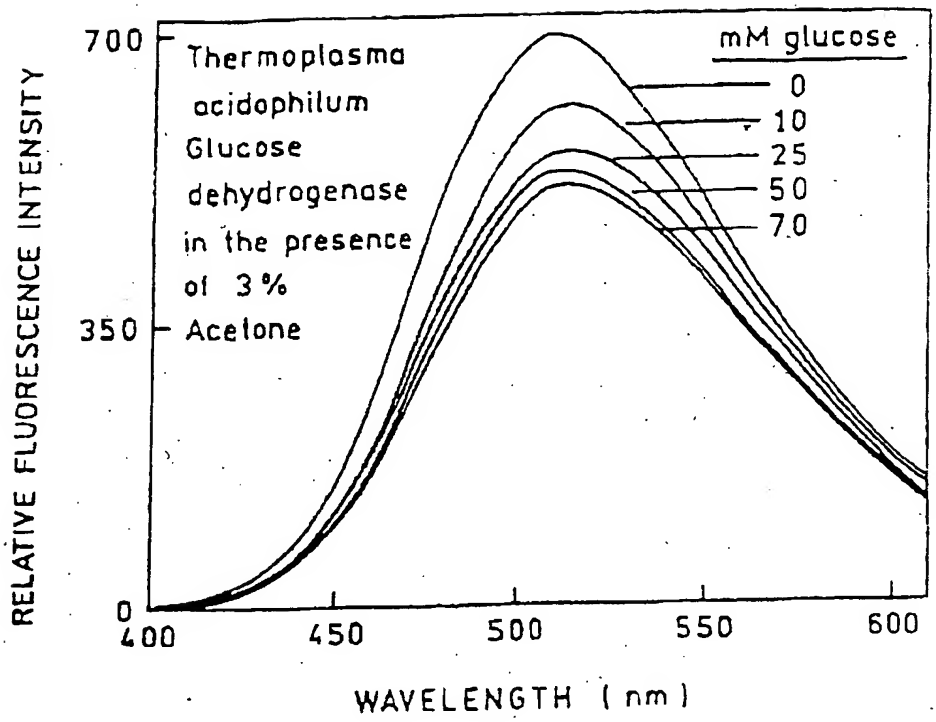


Figure 9. Emission spectra of ANS-labeled GD in the presence of 3% acetone and at different concentrations of glucose. [GD] - 3 μM. [ANS] = 4μM. Increase of glucose concentration over 70 mM does not introduce further changes in fluorescence intensity.

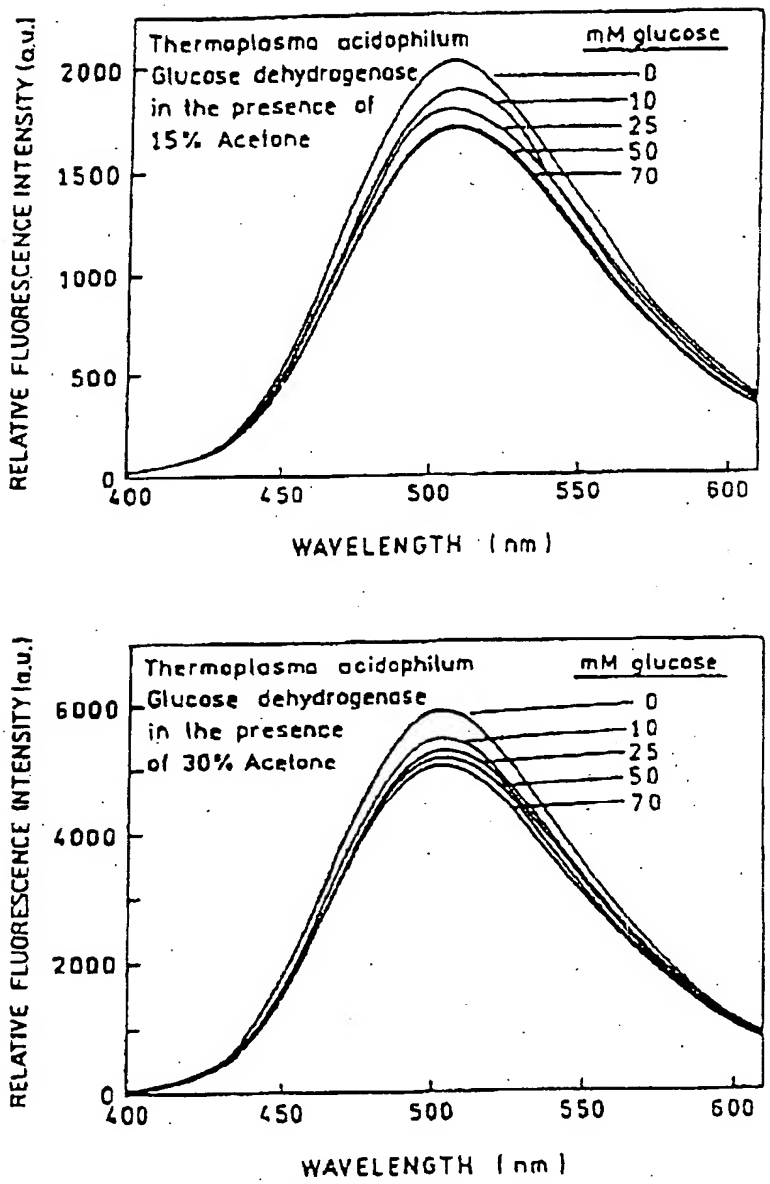


Figure 10. Emission spectra of ANS-labeled GD in the presence of 15% acetone (top) and 30% acetone (bottom) and at different concentrations of glucose. [GD] = 3 μM. [ANS] = 4 μM. Increase of glucose concentration over 70 mM does not introduce further changes in fluorescence intensity.

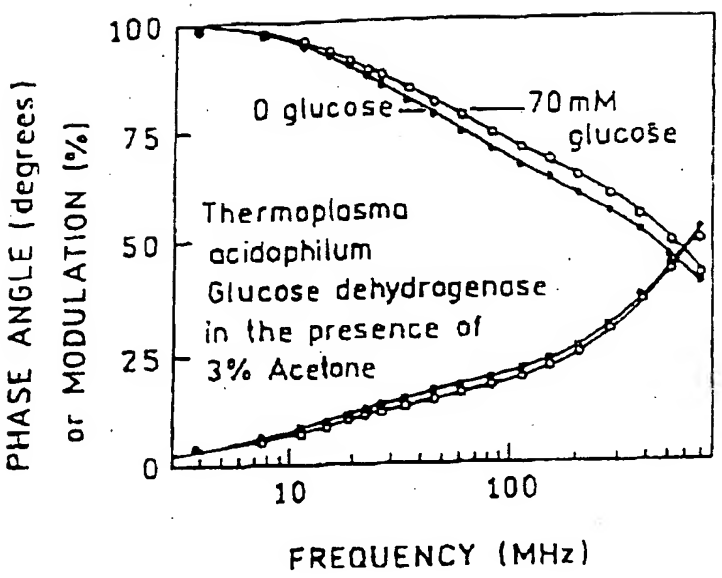


Figure.11. Frequency-domain intensity decay of ANS-labeled GD with 3% acetone in the absence and presence of glucose.

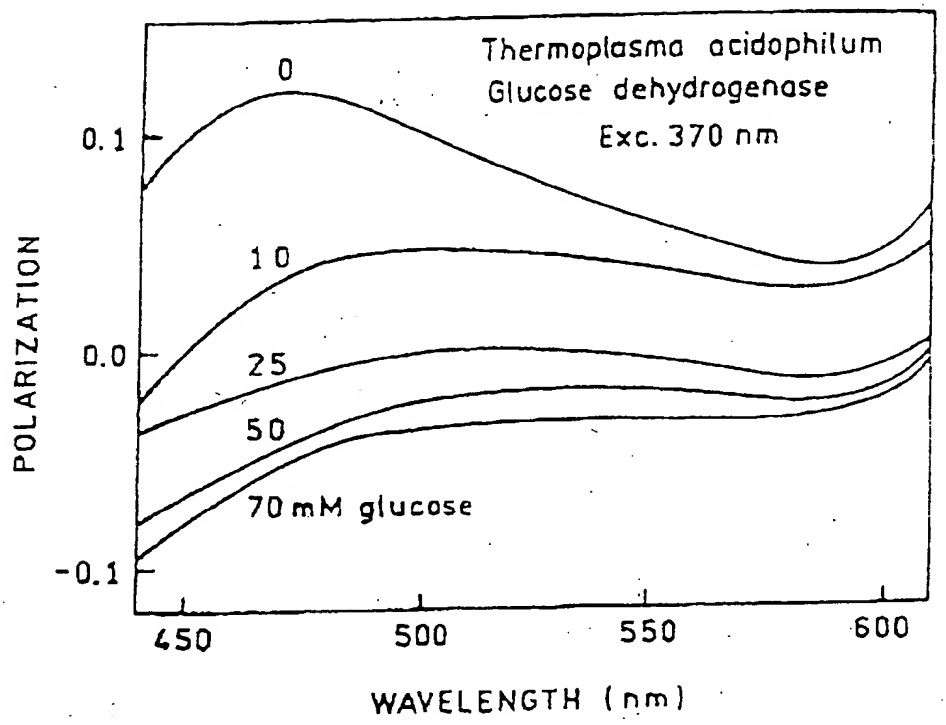


Figure 12. Polarization spectra of ANS-labeled GD in the presence of 3% acetone, and at different concentrations of glucose. Excitation was at 370 nm. [GD] = 3 μM. [ANS] = 4 μM.

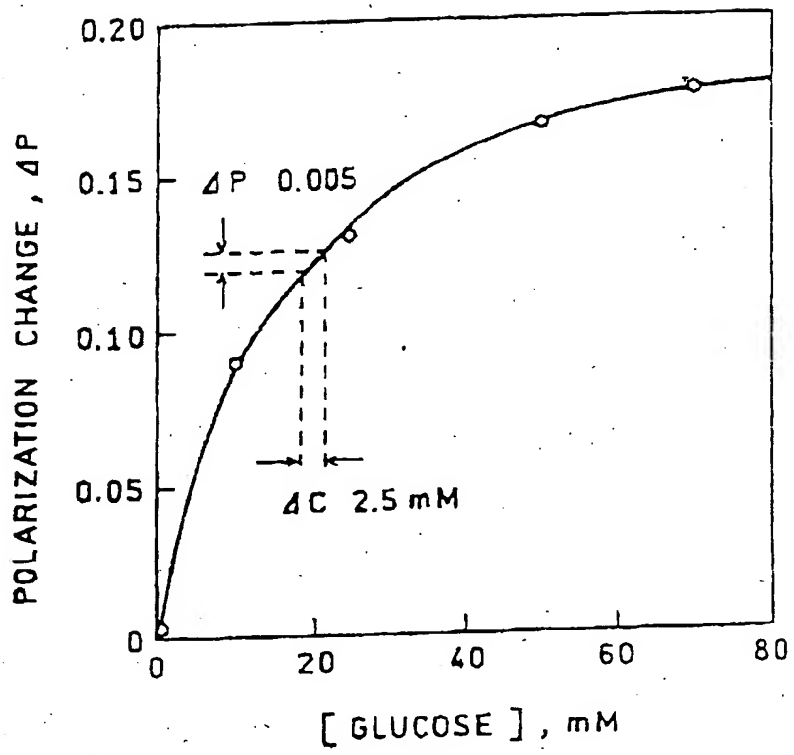


Figure 13. Effect of glucose on the polarization of GD in the presence of 3% acetone. The excitation was at 370 nm, and the emission was recorded at 470 nm. [GD] = 3μM. [ANS] = 4 μM.

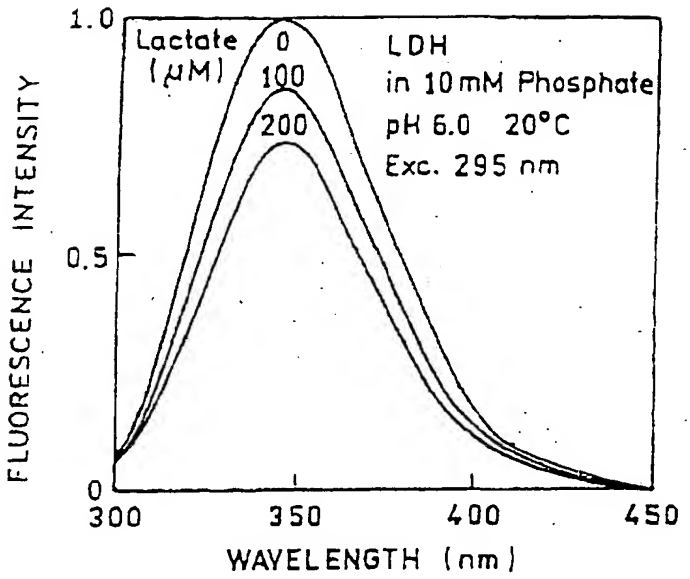


Figure 14. Intrinsic fluorescence of LDH in the absence and presence of lactate.

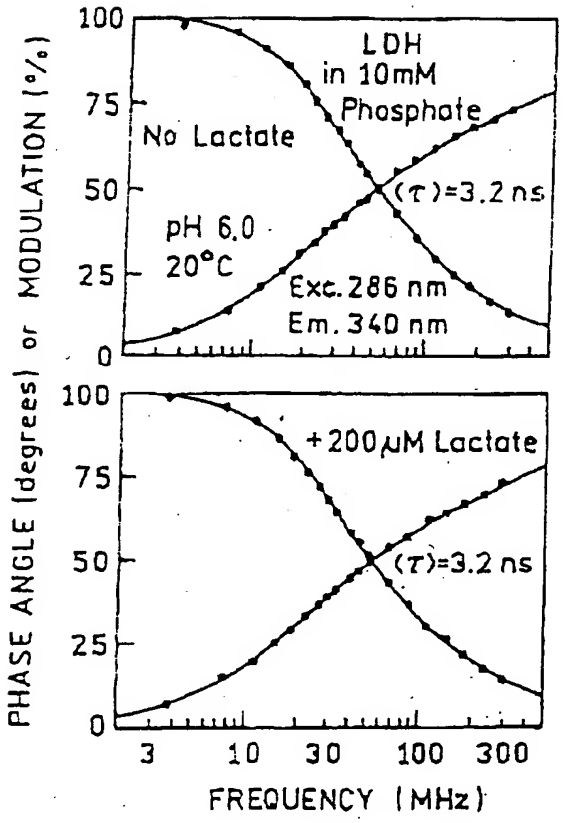


Figure 15. Frequency-domain intensity decay of the intrinsic fluorescence of LDH in the absence and presence of lactate.

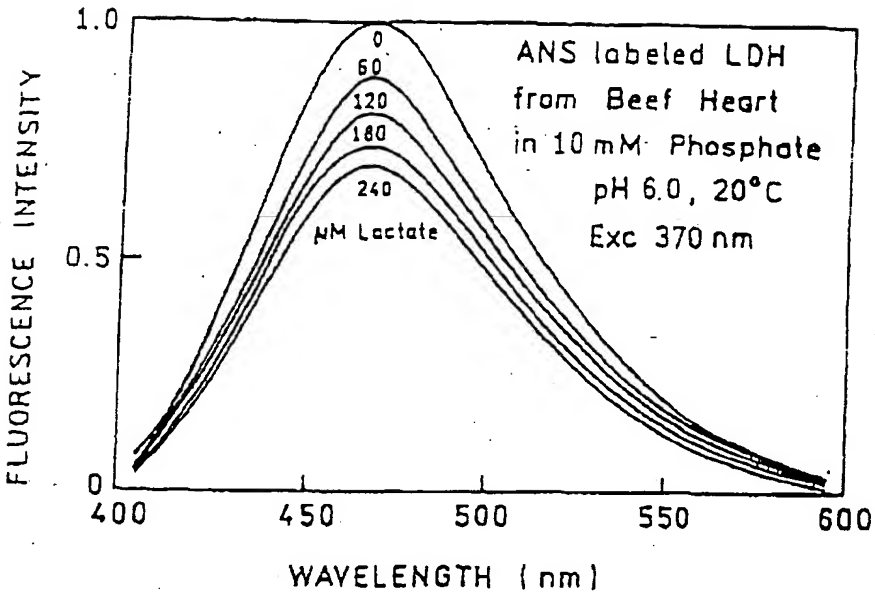


Figure 16. Emission spectra of ANS-labeled LDH in the presence of increasing concentrations of lactate. [LDH] = 3 μM. [ANS] = 4 μM.

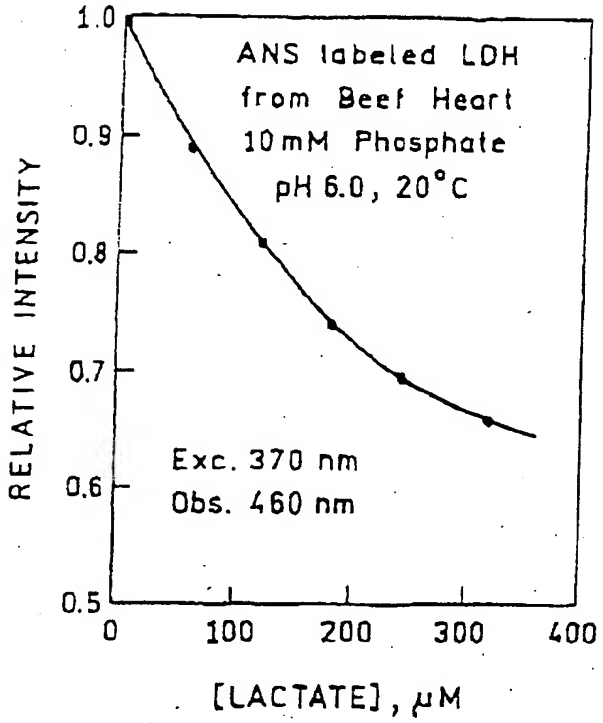


Figure 17. Relative emission intensity of ANS-labeled LDH in the presence of increasing concentrations of lactate.

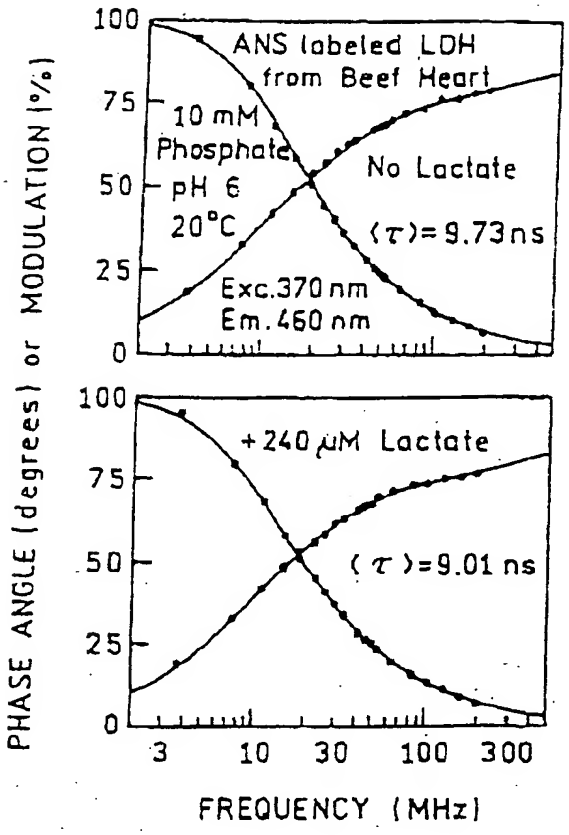


Figure 18. Frequency-domain intensity decay of ANS-labeled LDH in the absence and presence of lactate.

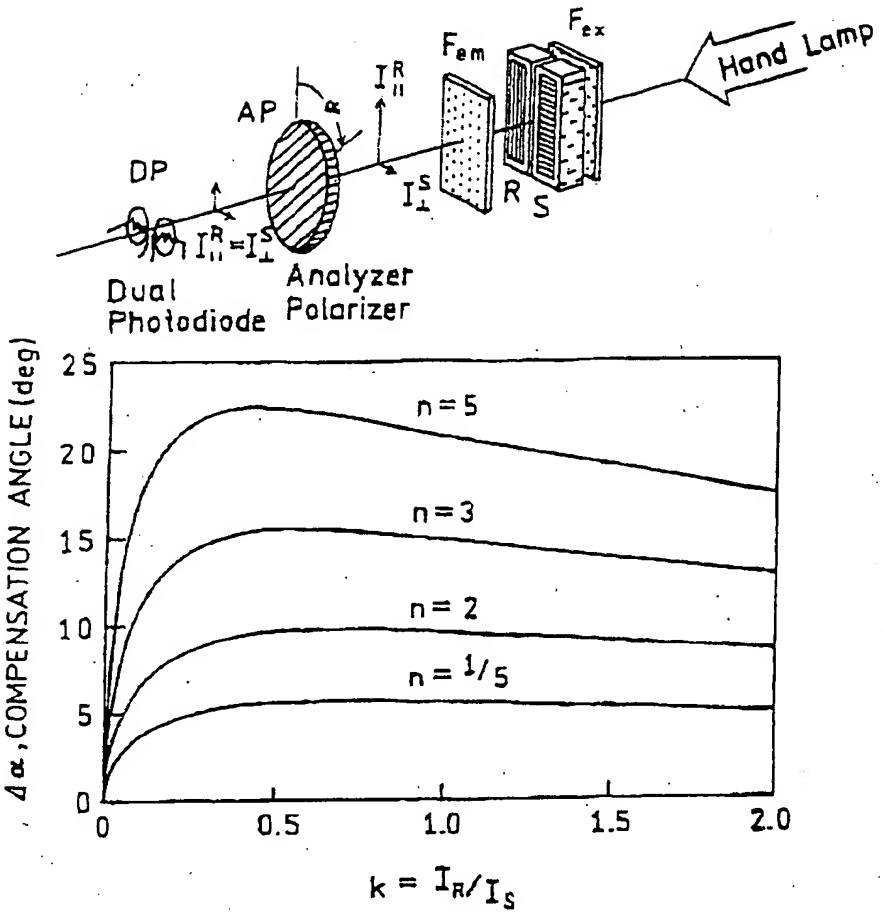


Figure 19. Schematic of polarization sensing (top) and simulations of the expected changes in compensation angle.

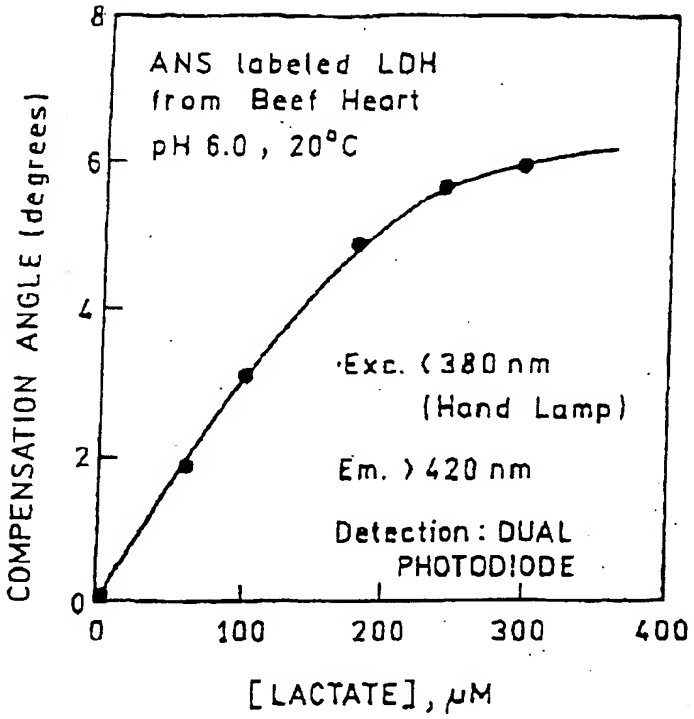


Figure 20. Polarization sensing of lactate bound on the emission intensity of ANS-labeled LDH.

INTERNATIONAL SEARCH REPORT

Intl . .ional Application No
PCT/US 00/24846

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/26 C12Q1/32 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data, EMBASE, CHEM ABS Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 356 780 A (ROBINSON GRENVILLE A ET AL) 18 October 1994 (1994-10-18) column 6; claims 1-4,7-9 column 4, line 29 - line 36 column 5, line 46 -column 6, line 2 column 3, line 59 -column 4, line 7 -/--	1-5, 7-15,18, 19, 21-25, 27-30, 33-37, 39-46, 48-50

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2001	21/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tuynman, A

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 00/24846

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUBE D ET AL: "DETECTION OF LIGAND-INDUCED CONFORMATIONAL CHANGES IN LACTATE DEHYDROGENASE USING FLUORESCENT PROBES" BIOKHIMIYA, vol. 52, no. 2, 1987, pages 179-187, XP000981451 ISSN: 0320-9725 abstract	1-10,14, 15,17, 21-29, 33-39, 42,43, 45,46,49
Y		20,33,51
A		31,32,51

Y	SMITH L D ET AL: "PURIFICATION AND CHARACTERIZATION OF GLUCOSE DEHYDROGENASE FROM THE THERMOACIDOPHILIC ARCHAEBACTERIUM THERMOPLASMA-ACIDOPHILUM" BIOCHEMICAL JOURNAL, vol. 261, no. 3, 1989, pages 973-978, XP000981445 ISSN: 0264-6021 cited in the application	20,33,51
A	abstract	1,16, 21-25, 30,33,47

P,X	D'AURIA SABATO ET AL: "The fluorescence emission of the apo-glucose oxidase from Aspergillus niger as probe to estimate glucose concentrations." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 263, no. 2, pages 550-553, XP002158779 ISSN: 0006-291X the whole document	1-13, 17-19, 33-44, 48,49,51

T	D'AURIA SABATO ET AL: "A thermophilic apoglucose dehydrogenase as nonconsuming glucose sensor" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 274, no. 3, 11 August 2000 (2000-08-11), pages 727-731, XP002158780 the whole document	1-13, 16-20, 33-44, 47-49,51

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 00/24846

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